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STUDIES ON THE NATURE OF THE
 α -ADRENERGIC RECEPTOR

by



UWE KURT TERNER

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on the Nature of the α -Adrenergic Receptor" submitted by Uwe Kurt Turner in partial fulfilment of the requirements for the degree of Master of Science.

Abstract

This thesis consists of two parts. In the first part, the effect of propranolol (a β -adrenergic blocking agent) on the α -adrenergic blockade induced by two

β -haloalkylamines was examined. Isolated rabbit aortic strips which were treated with phenoxybenzamine hydrochloride or dibenamine hydrochloride exhibited a partial recovery of response to norepinephrine and epinephrine following treatment with propranolol. This phenomenon has been examined to see whether this effect was due to (1) the displacement of the

α -adrenergic blocking agent from the α -adrenergic receptor site by the β -adrenergic blocking agent, propranolol; or (2) if the β -adrenergic blocking agent, by combining with the β -adrenergic receptors, unmasks those α -adrenergic receptor sites which have been 'spared' by the α -adrenergic blocking agent. By using varying concentrations of phenoxybenzamine hydrochloride and dibenamine hydrochloride in treating isolated rabbit aortic strips, it was found that at the higher concentrations of both α -adrenergic blocking agents, the degree of recovery following propranolol treatment was reduced when compared to strips which had been treated with lower doses of the α -adrenergic blocking agents. It was also observed that propranolol treatment did not reduce the radioactivity of strips which had been incubated with

^{14}C -dibenamine hydrochloride. It was concluded that the

β -adrenergic blocking agent, propranolol, acted by unmasking

those α -adrenergic receptor sites which had escaped blockade by the β -haloalkylamines.

In the second part of this thesis, the relationship was examined between the degree of protection of the

α -adrenergic receptor, conferred by different concentrations of epinephrine, and the uptake of ^{14}C -dibenzamine hydrochloride in protected and unprotected strips. Isolated rabbit aortic strips were treated with varying doses of epinephrine prior to ^{14}C -dibenzamine hydrochloride incubation. Tissues were then washed every 15 min for 3 hrs and then the sensitivity to a cumulative dose range of epinephrine was examined. As controls, the experiments were repeated with the omission of epinephrine. It was observed that significant α -adrenergic receptor protection could be obtained at a point when there was no observable difference in radioactivity between protected and unprotected rabbit aortic strips. At much larger concentrations of epinephrine, a significant count difference was obtainable along with very good receptor protection. As a result of these experiments, it was concluded that epinephrine probably protects sites other than the α -adrenergic receptor sites as well as α -receptor sites from combination with ^{14}C -dibenzamine hydrochloride, especially at higher concentrations. This study points out the necessity of always studying the relationship between receptor protection and count differences in protected and unprotected strips prior to embarking on receptor labeling studies.

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To my wife, Gail, and son, Lance, I wish to express my deepest gratitude for their patience, understanding, and support, without which this study would not have been possible. I wish to express my thanks by dedicating this thesis to both my wife and my son.

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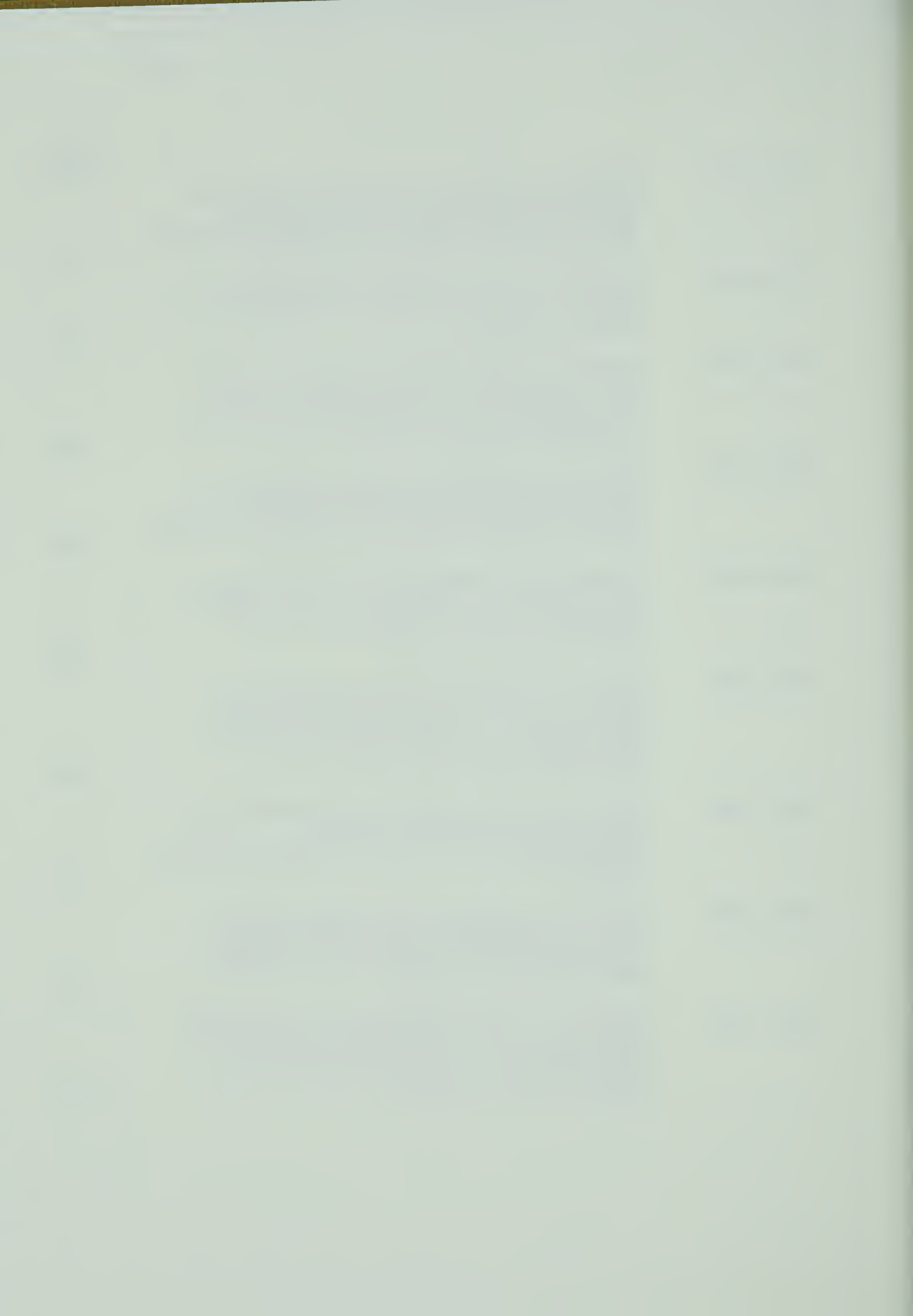
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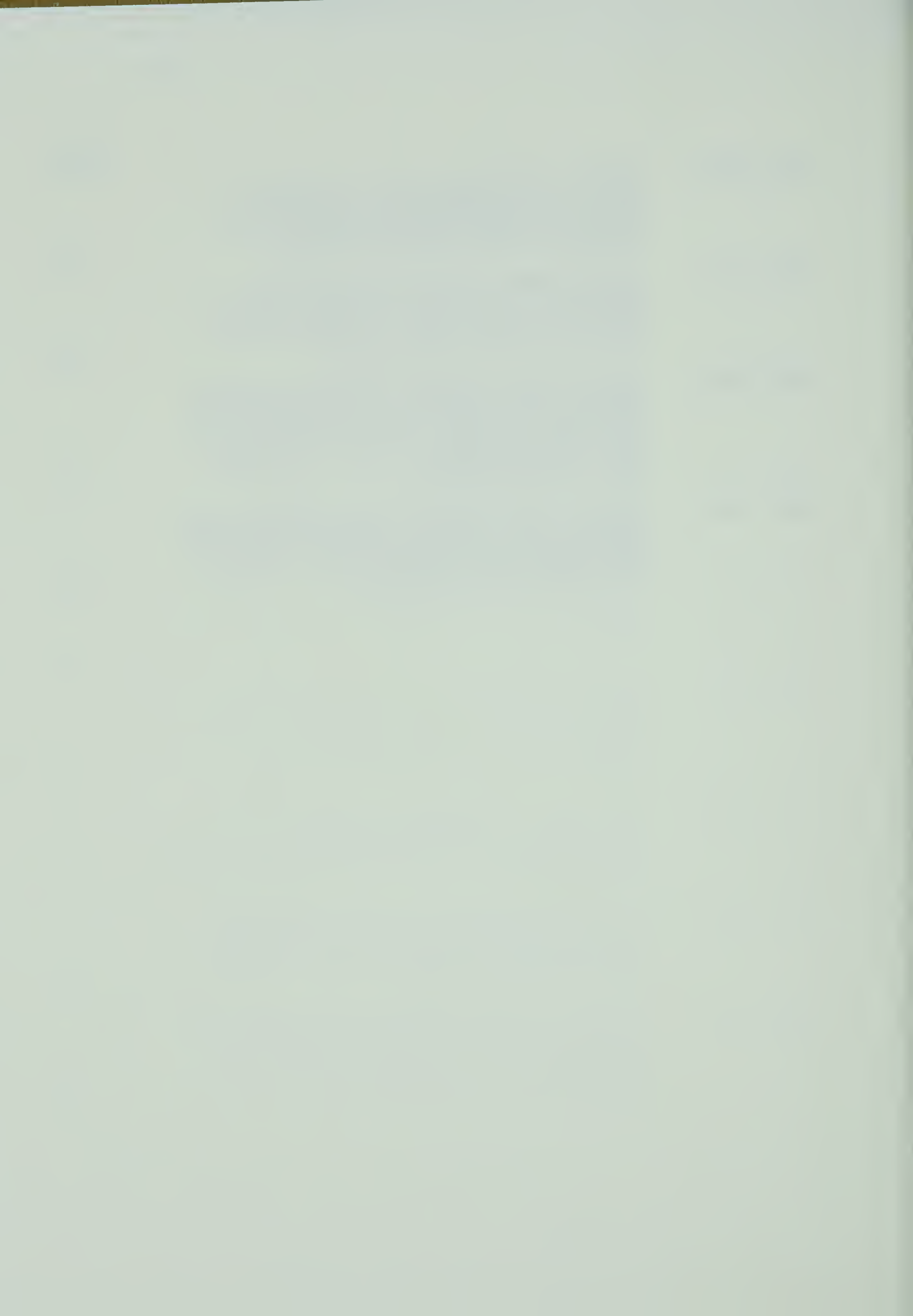


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CHAPTER I GENERAL INTRODUCTION



A. THE CONCEPT OF RECEPTORS

It has been assumed for many years that most drugs exert their actions via attachment to specific, chemically defined structures in the tissues on which they act. This concept was based on the high structural specificity which characterized the actions of many drugs and on the dose-response relationships in the action of mutually antagonist drugs.

Langley (1878) after studying the antagonist effects of atropine to pilocarpine in the cat submaxillary gland postulated that "there is some receptive substance with which both atropine and pilocarpine are capable of forming compounds according to some law in which their mass and chemical affinity for the substances are factors."

When Ehrlich (1900) considered the antagonist effects of atropine to the pilocarpine response, he came to the conclusion that the interactions of these drugs and their receptive substances do not involve the formation of a chemical bond, but is "loose and reversible". He postulated that the active drug and the antagonist must have similar "anchoring groups" permitting attachment to the same receptor and that differences in the two alkaloids are responsible for the differences in their physiological effects.

In later years, Clark (1926), proposed a theory of drug receptor interaction based on the Langmuir adsorption isotherm which attempted to provide a quantitative explanation for the relationship between the dose of a drug and the

biological response. In this theory the following assumptions were made: (1) drug molecules combine with receptors at a rate proportional to the concentration of free drug and to the number of free receptors; hence the magnitude of response would be directly related to the number of receptors occupied by an active drug (agonist); (2) at each receptor an "all or none" drug response is elicited by the agonist; (3) the occupation of one receptor does not alter the tendency of other receptors to become occupied; (4) drug and receptor exhibit a rigid "lock and key" relationship. Thus, according to Clark, in order to get maximum response, all the receptors would have to be occupied.

This theory does explain many of the features of the interactions of drugs with tissues but fails to explain why some drugs are agonists and other antagonists. Furthermore, it cannot account for the existence of compounds termed "partial agonists" that produce a biological response but are incapable of eliciting the maximum response seen with normal agonists.

The following observations indicated that all receptors did not have to be occupied in order for a maximum biological response to be elicited: Nickerson (1956), after treating guinea pig ileum with an appropriate dose of GD-121 (an irreversible blocking agent) demonstrated that a shift in the dose-response curve for histamine by two log units (100-fold) to the right was observed without any significant

decrease in either the slope or the maximum of the curve. This finding showed that maximal response could be achieved when only a small percentage of the available receptors were occupied. Furchgott (1954,1955) was also able to show that a maximum response to epinephrine could be achieved in rabbit aortic strips when some of the receptors were irreversibly blocked by dibenamine.

Stephenson (1956) reinvestigated Clark's dose-response relationships and concluded that: (1) a maximum effect can be produced by an agonist when only a small percentage of the total number of receptors is occupied; (2) the response is not linearly proportional to the number of receptors occupied; (3) different drugs may have different capacities to induce a response and consequently occupy different proportions of the receptors when producing equal biological responses. Stephenson also introduced the concept of "efficacy" as a result of his investigations. He defined efficacy as being inversely proportional to the number of receptors that have to be activated by the drug in order to induce a certain stimulus and to obtain a certain biological response.

Ariens (1954,1964) modified Clark's theory in a similar manner. He introduced two concepts: "affinity" which he defined as the ability of the drug to combine with the receptor, and "intrinsic activity", - the ability of a drug-receptor complex to induce a biological response. The concepts of affinity and efficacy are particularly useful

in differentiating the actions of "antagonists", "partial agonists" and "agonists" exerting their effects at identical receptors. Agonists have a high efficacy at a given receptor, competitive antagonists have zero efficacy, and partial agonists exhibit intermediate efficacy. Thus Stephenson defines partial agonists as drugs that induce submaximal biological responses even when occupying nearly all receptors, but, because of their relatively high affinity, diminish the action of agonists with high efficacy when the latter are added simultaneously.

Paton (1961) introduced his "rate theory" to explain drug-receptor interaction. He postulated that drug effects are proportional to the rate of drug-receptor combination, and not related to the proportion of receptors occupied. Each drug-receptor association would provide one "quantum" of excitation. According to this theory the value of the equilibrium constant (K_e) determines the potency of the drug, whereas the value of the dissociation rate constant (K_d) determines whether the drug is an agonist (having a high K_d), a partial agonist (an intermediate K_d), or an antagonist (a low K_d). Currently, it is uncertain whether the "rate theory" or the "occupation theory" is a better approximation of the real mechanism of drug-receptor interaction.

B. THE ADRENERGIC RECEPTORS

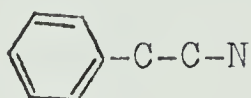
1. Classification

Dale (1906), in his classical work on the influence of ergot alkaloids on the effects of epinephrine and sympathetic nerve stimulation, was the first person to clearly differentiate two distinct types of receptors for epinephrine. Combination of epinephrine with one type of receptor usually led to an excitatory response and these receptors were "paralyzed" by ergot. Combination of epinephrine with the other group of receptors usually led to an inhibitory response and these receptors were unaffected by the ergot alkaloids. Ahlquist (1948) classified adrenergic receptors on the basis of the order of potency of five sympathomimetic amines (epinephrine, norepinephrine, isoproteranol, α -methyl-epinephrine, and α -methyl-norepinephrine) in several tissues. (It was found that the order of potency in one type of receptor was epinephrine > norepinephrine > α -methyl-norepinephrine > α -methyl-epinephrine > isoproteranol. On the other receptor, the following order of potency was demonstrated: isoproteranol > epinephrine > α -methyl-epinephrine > α -methyl-norepinephrine > norepinephrine. Hence it was concluded that there were two distinct types of adrenergic receptors. The α -adrenergic receptor is associated with most of the excitatory functions and one important inhibitory function ie. intestinal relaxation. The β -adrenergic receptor is associated with most of the inhibitory functions and one excitatory function ie. myocardial

stimulation. This classification was further supported by the fact that responses mediated through the α -receptor, but not the β -receptor could be blocked by the then known adrenergic blocking agents. For this reason these blocking agents were designated α -adrenergic blocking agents. Approximately a decade elapsed before a group of compounds were discovered which could specifically block β -receptors. Lands (1952) proposed a similar system of classification but the β -receptors of Ahlquist were subdivided into two groups.

2. Receptor Models

Berger and Dale (1910) after studying the structures of various sympathomimetic amines, came to the conclusion that for a compound to be active it had to possess a chain of two carbon atoms between an aromatic ring and an amino group. Hence the structure shown below was thought to fit the adrenergic receptor



Easson and Stedman (1933) compared the biological responses elicited by the optical isomers of epinephrine. It was found that the l-isomer had a great deal more activity than did the d-isomer. As a result of these findings they proposed that the greater pharmacological activity of l-epinephrine was due to the attachment of phenyl, β -hydroxyl and amino groups to specific areas of the receptor (Fig. 1A).

It was felt that the lower activity of the d-epinephrine was due to the inability of the β -hydroxyl to bind to the receptor (Fig. 1B). This idea was supported by the fact that the drug epinine (which does not possess a β -hydroxyl group) showed similar pharmacological activity to d-epinephrine (Fig. 1C).

Belleau (1958) introduced a model of the adrenergic receptor based on the examination of the structure-activity relationships of many analogues of epinephrine, and α -adrenergic blocking agents of the β -haloalkylamine type. He postulated that the receptor (Fig. 2A) had three important combining sites: (1) an 'A' site consisting either of a phosphate or carboxyl group which combined with the protonated nitrogen atom of epinephrine; (2) a 'B' site, which was flat and onto which the phenyl ring attaches; (3) M and P sites to which the meta and para-hydroxyl of the benzene would attach. It was suggested that the ethylenimmonium ion (Fig. 2B) of the β -haloalkylamines (Fig. 2C) would combine covalently with the 'A' site and thus produce prolonged blockage of the receptor.

Bloom and Goldman (1966) visualized the adrenergic receptor as a magnesium-activated adenosinetriphosphatase-adenosinetriphosphate (ATPase-ATP) complex. They postulated that agonists act by facilitating ATP utilization. In their view, the receptor is destroyed by interacting with a catecholamine, and subsequently regenerated by the binding of another molecule of ATP. The catecholamines were visualized

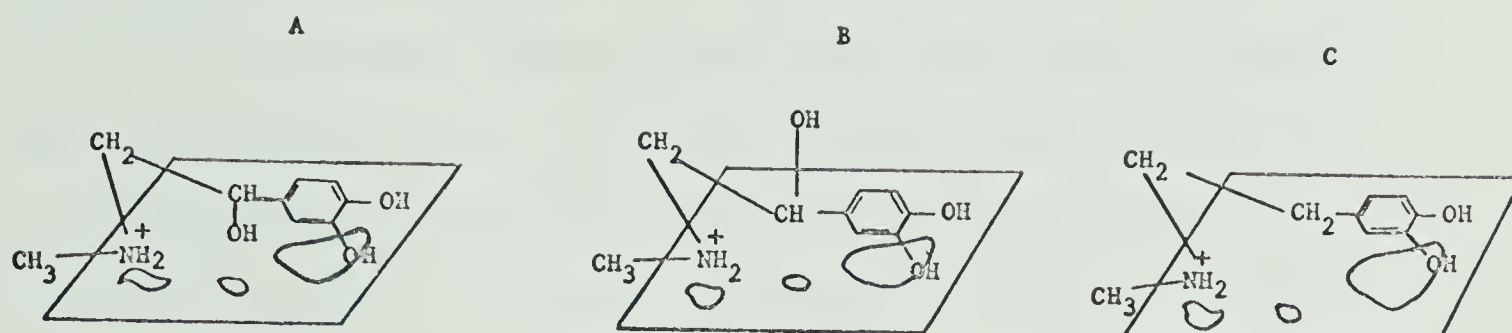


Fig. 1. Interaction of sympathomimetic amines with the postulated receptor sites. A. l-epinephrine; B. d-epinephrine; C. deoxyepinephrine (epinine).

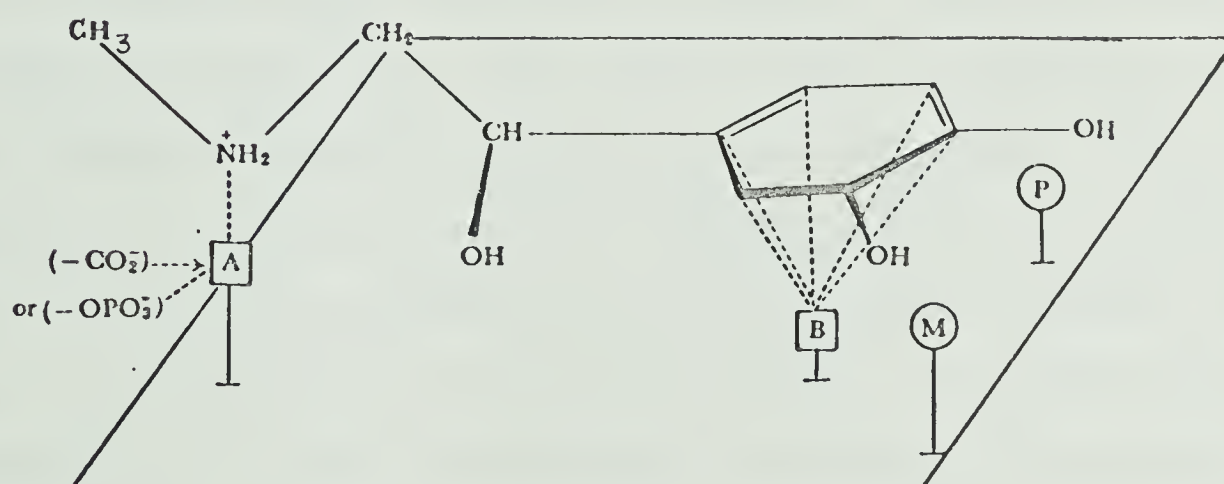


Fig. 2A. The interaction of epinephrine with the α -adrenergic receptor: A - anionic site; B - site of interaction of phenyl ring; M, P - sites of binding of phenolic groups (Belleau 1958)

as catalysts of ATP breakdown while antagonists of the β -haloalkylamine type were believed to inhibit utilization of the substrate, ATP.

Robison, Butcher and Sutherland (1967) proposed that particular sites on adenylyl cyclase are the α - and β -adrenergic receptors. This regulatory enzyme, a lipoprotein, is considered to exist in the cell membrane. It is depicted as consisting of a regulatory subunit, which faces the exterior of the cell, and an interior catalytic subunit. It is suggested that catecholamines interact with the regulatory subunit to influence the activity of the active site on the catalytic subunit (Fig. 3A). They found that the β -adrenergic agonists greatly increase the activity of adenylyl cyclase and this effect can be blocked by the β -adrenergic antagonists. One possibility considered was that the α - and β -receptors were located on separate regulatory subunits of adenylyl cyclase (Fig. 3B), both of which influenced the same catalytic subunit. Robison et al. suggested that combination of catecholamines with the α -adrenergic receptor would result in a decreased activity of adenylyl cyclase, and hence in contraction of the smooth muscle. On the other hand, combination with the β -adrenergic receptor was considered to result in increased adenylyl cyclase activity and hence, relaxation of the smooth muscle. Another possibility considered was that the α - and β -adrenergic receptors are bound to

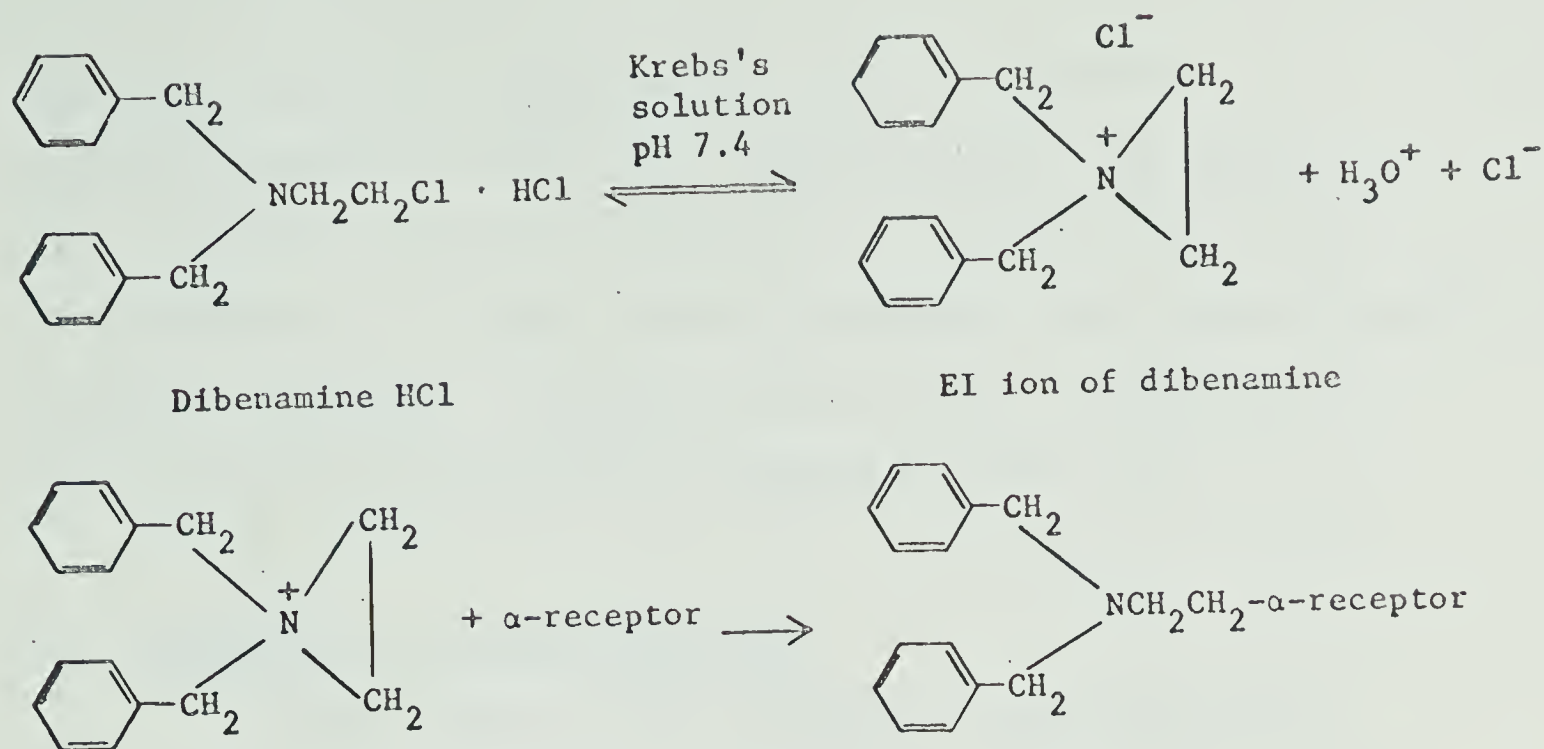


Fig. 2B. Formation of Ethylenimmonium (EI) ion from dibenamine and its reaction with the α -adrenergic receptor.

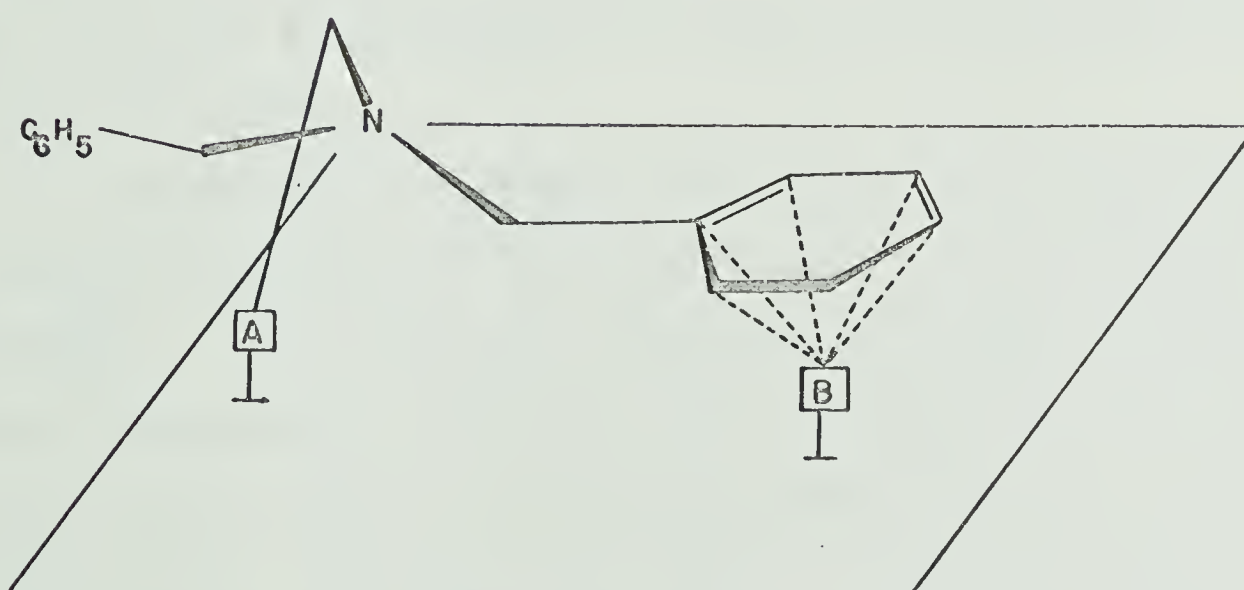


Fig. 2C. Combination of the β -haloalkylamine with the α -adrenergic receptor. (Belleau 1958)

separate catalytic subunits which may be located in different parts of the cell. Interaction of the agonists with both the α - and β -adrenergic receptors could lead to stimulation of adenylyl cyclase activity, and compartmentalization is invoked to explain those cases in which the α - and β -adrenergic effects are opposite (Fig. 3C).

C. ATTEMPTS TO ISOLATE RECEPTORS

There seems to be a paradox in pharmacology in that a great deal is known about drugs and yet very little is known about the portion of the cell (receptor) with which the drug interacts to give it's biological response. Since many of the concepts in pharmacology are based on the idea of specific receptors with which the various agonists and antagonists interact it is important to actually demonstrate their existence by attempting to isolate and identify the receptor material.

One type of approach which has been used extensively in the identification of the active centre of the enzymes is as follows: (1) the active centre of the enzyme is labeled with an irreversible inhibitor; (2) hydrolysis of the enzyme to amino acids follows and the amino acid-inhibitor complex is identified. The amino acid which is bound to the inhibitor is considered to be part of the active site in the intact enzyme molecule. In this manner Schaffer, May and Sumneron (1954) were able to show that the active centre of chymotrypsin,

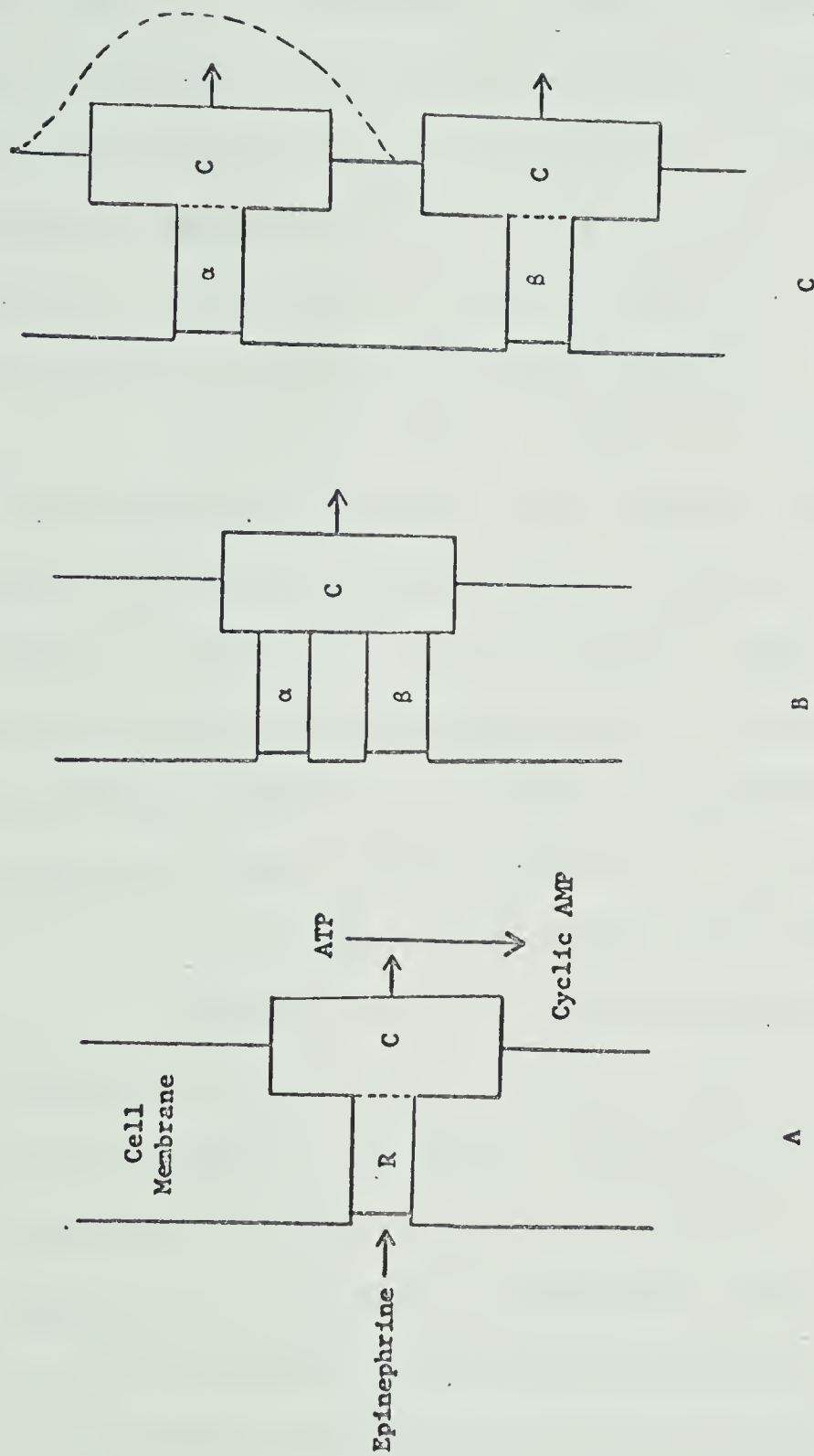


Fig. 3. Model of adenylyl cyclase as related to adrenergic receptors (Robison et al., 1967). A, model of adenylyl cyclase; R, regulatory subunit; C, catalytic subunit. B. α - and β -receptor as two different regulatory subunits bound to one catalytic subunit. C. α - and β -receptors as two different regulatory subunits bound to separate catalytic subunits.

after being labeled with ^{32}P -diisopropylfluorophosphate (^{32}P -DFP), contained serine, to which the DFP had complexed. Similar studies by other workers have demonstrated, by studying the amino acid sequences of active centres of various esterases and proteases, that DFP is always attached to serine. It was felt that the catalytic activity of the particular site may actually be controlled by the amino acid sequence surrounding serine. This type of approach may be very useful in trying to isolate portions of drug receptors.

Waser and Lüthi (1956) injected ^{14}C -curare or ^{14}C -decamethonium in minimal lethal doses into the mouse and then examined different areas of the diaphragm by autoradiographic technique. By this means, they demonstrated that the radioactivity was concentrated in the areas of the neuromuscular junction. Waser (1961, 1966) demonstrated, by the same technique, that ^{14}C -muscarone and ^{14}C -carbachol are bound to the motor endplate and other cholinergic receptors.

Chagas et al. (1956) demonstrated that an aqueous extract of electroplax formed a non-dialyzable complex with curare. Hasson and Chagas (1959) followed this study up by fractionating these aqueous extracts in order to identify the compound or components responsible for the fixation of curare and other related quaternary ammonium compounds. Using ^{14}C -gallamine triethiodide, which formed a non-dialyzable complex with the extract, these workers concluded that the component responsible for binding of quaternary ammonium

compounds is an acid mucopolysaccharide.

Ehrenpreis (1959) showed that there was a striking parallellism between the intact electroplax and an aqueous extract of electroplax in their ability to bind curare and other quaternary ammonium compounds. He obtained a supposedly electrophoretically pure fraction (1960) which he thought to be the acetylcholine receptor. However it must be pointed out that the fact that one obtains macromolecules complexing to drugs does not necessarily mean that the macromolecule is the receptor (Beychok, 1965). Also, it is important to point out that the bonds formed between the curare-like compounds and the receptor protein are of non-covalent nature and hence, the complex may dissociate before isolation is complete.

Wooley and Gommi (1964) demonstrated that they could selectively destroy the 5-hydroxytryptamine (5-HT) receptor of the rat stomach or uterus by treatment with purified neuramidase and ethylenediaminetetraacetate (EDTA). They also showed that the sensitivity of the tissue could be restored by treatment with a crude lipid extract of normal rat stomach or a purified ganglioside. Hence, they postulated the 5-HT receptor to be a ganglioside. These results could not, however, be duplicated by Offermeier and Ariens (1966).

Furchgott (1954) identified four distinct sets of contraction receptors in the smooth muscle of rabbit aorta, which are specific for adrenergic drugs, 5-hydroxytryptamine, histamine, and acetylcholine respectively. He showed that when this preparation is exposed to epinephrine bitartrate (1×10^{-4})

for 5 minutes and dibenamine hydrochloride (3×10^{-6}) added, the latter drug combines with the 5-hydroxytryptamine, histamine, and acetylcholine receptors, respectively, while the adrenergic receptor is protected, at least partially, from combination with this drug. Gaddum (1962) suggested that this method could be used to mask the 5-hydroxytryptamine, histamine, and acetylcholine receptors with the unlabeled dibenamine hydrochloride; the adrenergic receptor which would be protected under these conditions could then be labeled with radioactive dibenamine hydrochloride.

Dikstein and Sulman (1965a, 1965b) proposed that phospholipids could be the receptor for epinephrine in rabbit aortic strips. They based their conclusions on the following evidence: (1) receptors of aortic strips could be inactivated by treatment of the strips with 25% acetone for 2 minutes and it was observed that the sensitivity of the tissue to epinephrine could be restored by the addition of phospholipids; (2) using Gaddum's procedure and a modification of this procedure to label the strips, these workers demonstrated that greater than 80% of the total radioactivity of the aortic strips was located in the lipid fraction of isolated aortic strips. Hence they concluded that the adrenergic receptor is located in the lipid fraction of the tissue. Subsequently they produced evidence that epinephrine inhibits the uptake of ^3H -dibenamine hydrochloride into the cephalin fraction of rabbit aorta and thus suggested that cephalin is the α -adrenergic receptor.

Yong, Parulekar, Wright and Marks (1966) used ^{14}C -dibenamine hydrochloride to label the α -adrenergic receptor and to study the distribution of this drug in rabbit aortic tissue. It was found that approximately 20% of the total radioactivity was in the lipid fraction and the remainder was in the non-lipid residue. Also, upon examination of the components of the lipid fraction, no evidence could be found to support the claim that cephalin serves as a receptor for dibenamine hydrochloride. Clearly, from these results, the non-lipid residue cannot be eliminated in the search for the α -adrenergic receptor as implied by Dikstein and Sulman.

Lewis and Miller (1966) used ^3H -phenoxybenzamine hydrochloride as the irreversible α -adrenergic blocking agent to label the α -adrenergic receptors of rat seminal vesicles according to a modification of Gaddum's procedure. They then examined the binding of ^3H -phenoxybenzamine hydrochloride to the lipid and non-lipid fractions. From their results, they concluded that the α -adrenergic receptors were not located in the lipid fraction.

Takagi and Takahashi (1968) used Gaddum's approach to label the acetylcholine receptors in the dog ileum with ^3H -dibenamine hydrochloride after having protected it with atropine. After fractionating the tissue they isolated a ^3H -dibenamine-protein complex and suggested that the receptor is protein in nature.

D. STATEMENT OF THE PROBLEM

Many workers have followed the suggestions of Gaddum (1962) which were based on the receptor protection experiments of Furchgott to label the α -adrenergic receptor with the goal of isolating and identifying the antagonist-receptor complex.

Yong and Marks (1966) attempted to label the α -adrenergic receptors of rabbit aortic strips with ^{14}C -dibenamine hydrochloride according to the procedure suggested by Gaddum. It was anticipated that strips protected with epinephrine would be radioactive while unprotected strips would contain negligible amounts of radioactivity. This expectation was based on the idea that a strip treated with unlabeled dibenamine hydrochloride in a concentrations sufficient to block all the pharmacological receptors would not take up any ^{14}C -dibenamine hydrochloride on subsequent exposure to this agent. However, this was clearly not the case, since it was found impossible to saturate the tissue after repeated exposures to dibenamine hydrochloride and a modification of the labeling procedure was required. It was decided to study the possibility of utilizing a simpler labeling procedure described by Dikstein and Sulman (1965b). The experimental design was as follows: a rabbit aortic strip was treated with epinephrine to protect the adrenergic receptors and ^{14}C -dibenamine hydrochloride added to react with the 5-hydroxytryptamine, histamine and acetylcholine receptors. The tissue was then washed with Krebs's bicarbonate solution to remove epinephrine and loosely bound ^{14}C -dibenamine

hydrochloride. A strip treated in this manner was designated as the experimental strip. As a control, the experiment was repeated with the omission of the protecting dose of epinephrine in the first step. By this procedure, it was anticipated that strips which were not protected by epinephrine would contain significantly greater radioactivity than strips which were protected. This was found to be the case and it was assumed that this difference in radioactivity represented that portion of the ^{14}C -labeled dibenamine hydrochloride combined with sites which might be in large part α -adrenergic receptor sites. According to Moran et al. (1967) there is no correlation between the degree of receptor protection provided by an agonist, such as epinephrine, and the count differences observed in protected and unprotected strips. This would result if an agonist protected large numbers of non-specific sites from combination with ^{14}C -dibenamine hydrochloride as well as the α -adrenergic receptor sites. For further progress in studies of this type, it is vital to know whether there is a relationship between receptor protection and count difference observed in protected and unprotected strips. For this reason the relationship between the degree of protection conferred by different concentrations of epinephrine, and the count differences observed, has been studied and is reported in Chapter IV of this thesis.

Olivares, Smith and Aronow (1967) demonstrated that α -adrenergic blockade produced by phenoxybenzamine hydrochloride

(an irreversible α -blocking agent) in dogs and isolated rabbit aortic strips could be almost completely reversed by the administration of the β -receptor blocking agent, propranolol. It was also shown by these workers that propranolol administration prior to the addition of phenoxybenzamine hydrochloride interfered with the establishment of α -adrenergic receptor blockade. It was concluded by these workers that propranolol interacts with a receptor previously blocked by α -adrenergic blocking agents such as phenoxybenzamine hydrochloride, and displaces them from the receptor. It was therefore proposed to test this idea as follows: A series of aortic strips would be labeled with ^{14}C -dibenamine hydrochloride in order to block the α -adrenergic receptors. Half of the strips would be treated with propranolol and the radioactivity of the propranolol treated strips compared with those which had not been treated with propranolol. If the theory of Olivares et al. was correct, it would be expected that propranolol treated strips would contain less radioactivity than strips untreated with propranolol. Work on this problem will be reported in Chapter III.

CHAPTER II METHODS AND MATERIALS

A. Drugs and Solutions.

^{14}C -dibenamine hydrochloride was prepared from benzyl-7- ^{14}C -chloride (0.25 mCi/mM) in this laboratory (Parulekar-1965) by a method previously used by Gump & Nikawitz (1950) to prepare the unlabeled compound. It had a specific activity of 0.48 mCi/mM. The radiochemical purity has been checked by reverse isotope dilution analysis (Yong-1968).

The Krebs's bicarbonate solution used in this study contained 0.116 M NaCl, 0.0046 M KCl, 0.0024 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.001 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.021 M NaHCO_3 and 0.045 M dextrose.

The McEwen solution used in this study contained 0.129 M NaCl, 0.0056 M KCl, 0.00214 M CaCl_2 , 0.0013 M NaH_2PO_4 , 0.025 M dextrose and 0.025 M NaHCO_3 .

The volume of solution (Krebs's or McEwen) in the organ bath was 14.8 ml. Drugs were made up to a final volume of 0.2 ml before addition to the bath. The following drugs were used: l-epinephrine bitartrate (Nutritional Biochemicals); l-norepinephrine hydrochloride (Fisher); dibenamine hydrochloride (SKF); phenoxybenzamine hydrochloride (SKF); propranolol hydrochloride (Ayerst); and ^{14}C -dibenamine hydrochloride. Stock solutions of l-epinephrine bitartrate (7.5 mg/ml) and l-norepinephrine hydrochloride (0.3075 mg/ml, 3.075 mg/10 ml) were freshly prepared in normal saline for each experiment and used to prepare a series of dilutions of this drug. Solutions of dibenamine hydrochloride, ^{14}C -dibenamine

hydrochloride and phenoxybenzamine hydrochloride were always freshly prepared 5 min before addition to the bath with normal saline containing 0.01 M HCl plus one drop of 95% ethanol to aid the dissolving of the drugs. All solutions were kept in an icebath for the duration of the experiment. Drug concentrations unless otherwise specified, are expressed in grams of salt per milliliter of bath fluid.

B. Preparation of Rabbit Aortic Strips.

Male New Zealand white rabbits of weight range 1.5 to 2.5 kg were killed and the descending thoracic aorta quickly removed. The aorta was placed in a Petri dish containing Krebs's bicarbonate solution aerated with 95% oxygen and 5% carbon dioxide, and cleaned of all excess adipose and connective tissue. Following the method of Furchgott & Bhadrakom (1953) the aorta was cut into a helical strip. The procedure is as follows: the aorta is held between the thumb and fingers of the left hand and gradually rotated and moved forewards toward a pair of sharp-pointed scissors held in the right hand to permit a continuous spiral incision at an angle of approximately 15° relatively to the long axis of the intact aorta. A cut strip (approximately 30 mm x 5 mm) was mounted in an organ bath (Fig. 4) containing Krebs's bicarbonate solution (15 ml) aerated with 95% oxygen and 5% carbon dioxide mixture to give a pH of 7.4. The temperature of the bath was maintained at 37° by circulation of warm water through the outer jacket

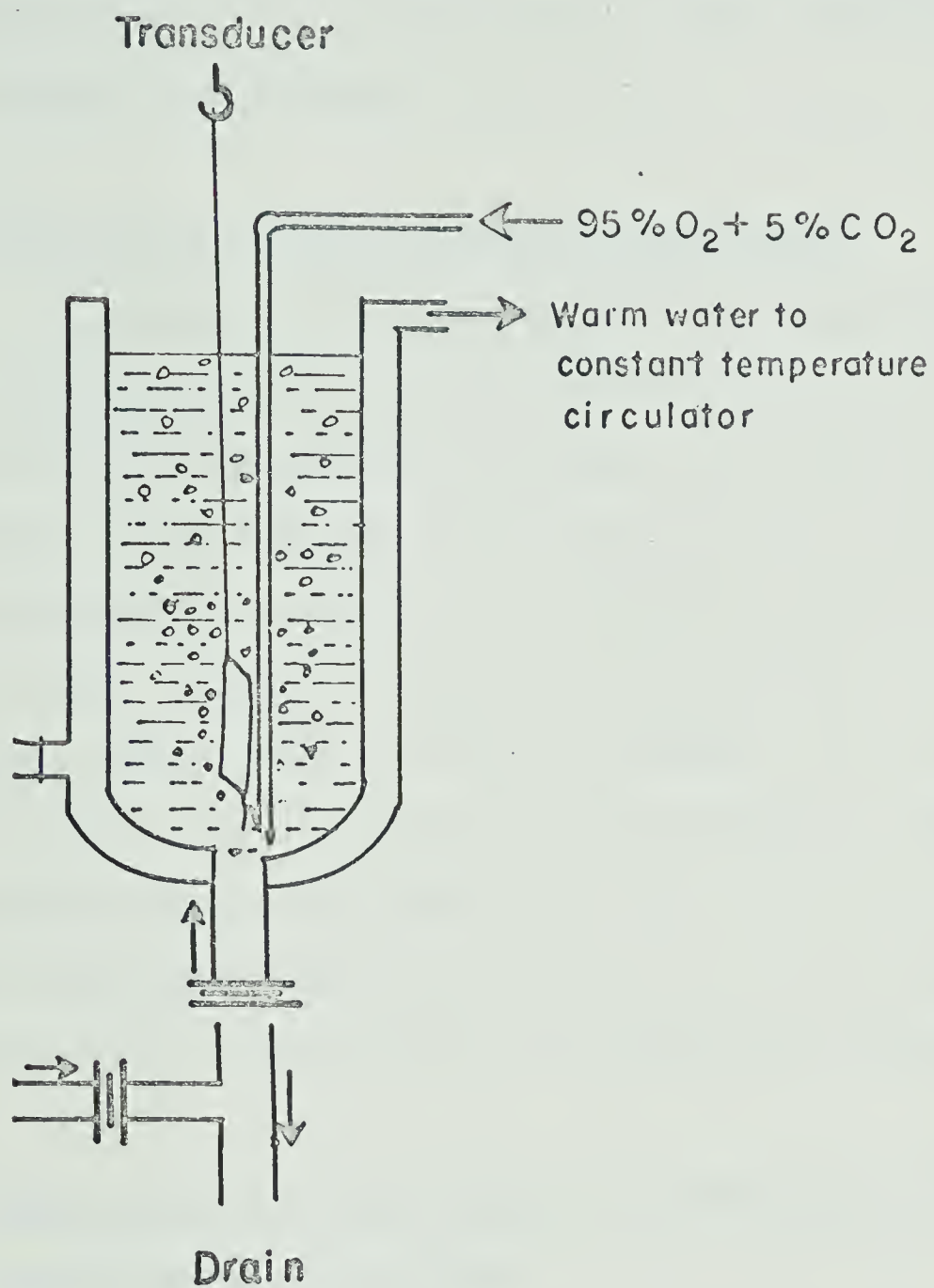


Fig. 4. Apparatus Used For Mounting Isolated Rabbit Aortic Strips.

of the organ bath by means of constant temperature circulator. The strips were placed under an initial tension of 2 g and allowed to relax for 2 hrs prior to being exposed to various experimental procedures.

C. Calibration of the Recording Instruments.

Aortic strips were mounted as single strips (Fig. 4) and contraction recorded by means of force and displacement transducers (Grass FT.03) connected to a Grass model 5Pl polygraph. The baseline was first set and the driver amplifier was calibrated to yield a 2 cm deflection per 200 millivolt input from a companion plug-in preamplifier. The transducer was then loaded with a series of weights of 1 to 4 g. The sensitivity of the instrument was adjusted so that a weight of 1 g produced an upward deflection of 1 cm of the pen. The response of the instrument was linear in the 1 to 4 g range. The chart speed was set at 0.25 cm/sec throughout the experiment.

D. Determination of the Degree of Protection of the α -Adrenergic Receptors.

The degree of protection afforded the α -adrenergic receptors by means of various doses of epinephrine was calculated by means of the following equation:

$$P = R^1/R \times 100$$

where P (the degree of protection) = percentage of the original response to epinephrine

R = Tension (g) developed by strips to a dose of epinephrine prior to the protecting dose of epinephrine and dibenamine (3×10^{-6})

R^1 = Tension (g) developed by strips to the same dose of epinephrine after the protecting dose of epinephrine and dibenamine (3×10^{-6})

The degree of protection was determined in the above manner with several doses of epinephrine and an average value was obtained.

E. Determination of Radioactivity in Tissue Samples.

(1) Preparation of Tissue Sample for Counting.

Aortic strips were stored in a frozen state until an adequate number accumulated (usually 16-20 samples). The tissues were then lyophilized and the dry weight determined. The tissues were then dissolved in 0.2 ml 5N KOH per 10 mg dry wt by warming to 70° for 1 to 2 hrs. Initially, we carried out this process in 10 ml graduated stoppered vials, but found that as a result of transferring aliquots from these vials to the liquid scintillation vials, a certain amount of error was introduced. A suggestion from Dr. D. Cook of this department led to the following approach: the aortic strips (dry wt approximately 8-12 mg) were placed directly in the scintillation vials, and 0.2 ml of 5N KOH was added. The vials were then heated in a water bath at 70° for 1 to 2 hrs. The vials were then allowed to cool and 5.3 ml reagent grade methanol was added followed by scintillation solution (12.5 ml). The scintillation solution was prepared by dissolving 6 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 2-p-phenylene-bis (5-phenyl oxazole) (POPOP) in one liter of reagent grade toluene. Each of the samples was counted for 40 min in a Nuclear Chicago

Unilux model 6850 liquid scintillation counter.

(2) Determination of Balance Point and Counting Efficiency of Liquid Scintillation Counter

The Unilux Liquid Scintillation Counter utilizes the same fixed discriminators in both upper and lower channels, but the energies that the discriminators are equivalent to depend on the attenuator setting in each channel. The attenuator settings and channel arrangements can be properly chosen so that the ratio of the counting rates in the two channels (channels ratio) will change in a way to be a measure of the counting efficiency or the degree of quenching. Using a series of samples of known activity and quenched in varying and known degrees, a curve can be constructed by plotting the counting efficiency versus the channels ratio for each quenched sample. This allows for direct reading of the counting efficiency from the curve as soon as the channels ratio has been measured.

A standard unquenched ^{14}C -toluene sample was used to determine the balance point in one of the analyzer channels of the Unilux Liquid Scintillation Counter. This was done by increasing the amplifier gain in this analyzer channel until the maximal number of counts (representing β -particles) are obtained. A region of gain values exists where the pulses observed do not change appreciably with a small change in gain. This region is the "balance point"; the amplifier gain is adjusted to provide highest efficiency or counting rate

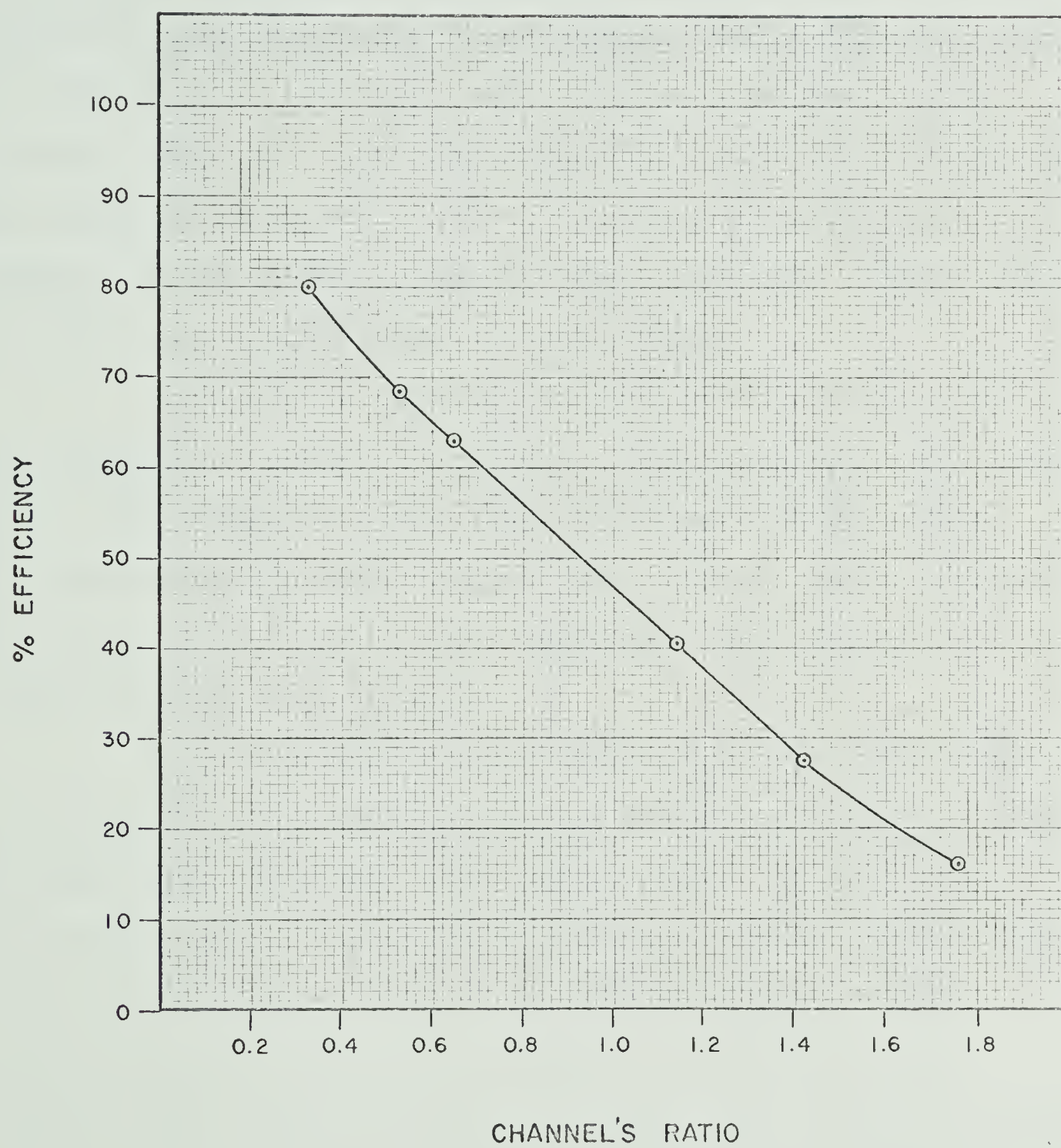


FIG. 5 CARBON-14 CHANNELS RATIO QUENCH CORRECTION CURVE

between the two discriminator levels for the unquenched ^{14}C -toluene standard. The second attenuator channel was then adjusted so that the number of pulses or counting rate was one third the value obtained in the first channel. A series of ^{14}C -toluene standards of known activities but with different degrees of quenching were then counted, their channels ratio and efficiencies obtained, and a quench correction curve plotted. (Fig. 5). This curve was then utilized to determine the counting efficiencies of the unknown samples after the channels ratio of the latter had been obtained. These results give the true counts (dpm) of each sample.

Each of the samples was counted for 40 min, and the background counted for 80 min. These periods were calculated to be long enough to bring the percentage standard error for the corrected count down to $\pm 2\%$. For the sake of brevity, the abbreviation dpm is used throughout this thesis for corrected counts per minute.

The responsiveness of the aortic strip to drugs decreases from the portion nearest the heart to the portion furthest from the heart. For this reason, after cutting the aortic spiral into four strips, the portion nearest the heart was denoted as Strip 1 and the remaining portions labeled consecutively as 2, 3, and 4. To reduce bias between experimental and control strips, the following strips were selected as experimental and controls in a series of experiments:

Experiment Number	Portions selected as Exp. strip	Portions selected as Control strip
1	1, 2	3, 4
2	2, 3	4, 1
3	3, 4	1, 2
4	4, 1	2, 3
5	1, 2	3, 4

CHAPTER III THE EFFECT OF PROPRANOLOL ON α -ADRENERGIC
RECEPTOR BLOCKADE ON RABBIT AORTIC STRIPS

The Effect of Propranolol on α -Adrenergic Receptor Blockade

Introduction

Current concepts in adrenergic pharmacology are based on the concept of two types of receptors at the adrenergic effector sites. These receptors are classified, according to Ahlquist (1948), into α and β types. This classification serves an important role in understanding adrenergic drug reactions. However, certain facts are difficult to reconcile with this simple classification of adrenergic receptors. For example, Hull et al. (1960) demonstrated the antagonism of various α -adrenergic blocking agents by the β -adrenergic blocking agent, dichloroisoproteranol. This observation was subsequently confirmed by Garrett et al. (1966), Gulati et al. (1965), and Sharma (1966), who employed different α - and β -blocking agents.

Although various hypotheses have been advanced to explain these observations, at the present time, no single explanation appears adequate to fully explain the phenomenon. Hull et al. (1960) suggested two hypotheses: 1) the α -adrenergic blocking agent is displaced from the receptor site by the β -adrenergic blocking; 2) the β -adrenergic blocking agent, by combining with the β -receptors, allows the revealing of those α -receptor sites which had escaped blockade by the α -adrenergic blocking agent. The studies of Gulati et al. (1965) with the rabbit aortic strip provided evidence for the first of these two hypotheses.

Olivares, Smith and Aronow (1967) showed that the phenoxybenzamine-induced blockade in dogs and isolated rabbit aortic strips could be reversed by the administration of propranolol. They also reported that prior administration of propranolol to dogs and isolated rabbit aortic strips interfered with the establishment of α -adrenergic blockade. From these results, it was suggested that the β -adrenergic blocking agent, propranolol, interacted with the α -adrenergic blocking agent, phenoxybenzamine hydrochloride, to displace the α -receptor blocking agent from its receptor site. These workers provided evidence which indicated that the second of the two hypotheses suggested by Hull et al. (1960) could not be correct. The difficulty in accepting the explanation of Olivares et al. (1967), resided in the fact that the β -haloalkylamine group of α -adrenergic blocking agents are believed to act by alkylating the receptor (Harvey and Nickerson, 1954), thus forming a firm covalent bond. It does not seem likely that this bond could be readily cleaved by the addition of propranolol.

It seemed possible to resolve this controversy by employing a ^{14}C -labeled α -adrenergic blocking agent of the β -haloalkylamine group, viz., ^{14}C -dibenamine hydrochloride. We could not use ^{14}C -phenoxybenzamine since it was not available at this time. What we proposed to do was to treat isolated rabbit aortic strips with ^{14}C -labeled dibenamine hydrochloride in a dose adequate to cause α -adrenergic receptor blockade. Then, after treating the tissue with propranolol we would examine the tissue to see if there was any reduction of radioactivity

and hence a reduction of bound ^{14}C -dibenamine hydrochloride. If a reduction of radioactivity would be demonstrated, it was then proposed to see whether a correlation existed between the loss of radioactivity and the recovery of sensitivity to norepinephrine. Our first experiments were directed at repeating the experiments of Olivares et al. (1967) using isolated rabbit aortic strips and phenoxybenzamine hydrochloride to see if we could duplicate them. Our second experiments were directed to seeing whether propranolol could reverse the α -adrenergic blockade produced by dibenamine hydrochloride in rabbit aortic strips. Finally, we carried out the studies with ^{14}C -dibenamine hydrochloride described above.

Experimental

(a) The Effect of Propranolol on Phenoxybenzamine-Induced α -Adrenergic Blockade.

Four rabbit aortic strips were mounted, as described previously, in four organ baths maintained at 37° and the tension of the strips adjusted to 2 g. The tissues were allowed to relax for approximately 2 hrs. The bath fluid used was McEwen solution and this was changed every 15 min. The contractile responses of strips were recorded after exposure to the following concentrations of norepinephrine: 4.1×10^{-10} , 4.1×10^{-9} , 4.1×10^{-8} , 4.1×10^{-7} , and 4.1×10^{-6} . The individual strips were then each treated in a different manner as described below:

Strip 1	Strip 2	Strip 3	Strip 4
1. Incubate with phenoxybenz-amine 1×10^{-7} for 10 min	Incubate with phenoxybenz-amine 1×10^{-7} for 10 min	Omit	Omit
2. Wash once	Wash once	Wash once	Wash once
3. Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range
4. Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr
5. Propranolol 1.48×10^{-5} for 10 min	Omit	Propranolol 1.48×10^{-5} for 10 min	Omit
6. Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range
7. Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr
8. Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range
9. Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr
10. Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range	Test with nor-epinephrine dose range

The data is recorded in Figs. 6A, 6B, 6E, and 6F.

The above procedure was repeated, but with a higher dose of phenoxybenzamine (1×10^{-6}). The data is recorded in Fig. 7A and 7B.

(b) The Effect of Propranolol on Dibenamine-Induced
 α -Adrenergic Blockade.

Experiments were carried out using the same plan as outlined in the phenoxybenzamine hydrochloride investigation, but with some slight modifications. Strips were incubated with dibenamine hydrochloride (3×10^{-6}) for 20 min in place of phenoxybenzamine hydrochloride (1×10^{-7}) for 10 min. The responsiveness of the strips was tested with epinephrine at concentrations of 1×10^{-8} , 1×10^{-7} , and 1×10^{-6} . The experiment was repeated twice. The data is recorded in Figs. 8A and 8B.

(c) The Effect of Propranolol on ^{14}C -Dibenamine-Induced
 α -Adrenergic Blockade.

Four aortic strips were mounted in constant temperature baths as previously discussed and allowed to relax for 2 hrs. Each strip was then exposed to cumulative doses of epinephrine 1×10^{-8} , 1×10^{-7} , 1×10^{-6} , and the responses recorded. The tissues were then washed with McEwen solution every 15 min until the strips relaxed.

The rabbit aortic strips were then treated as follows:

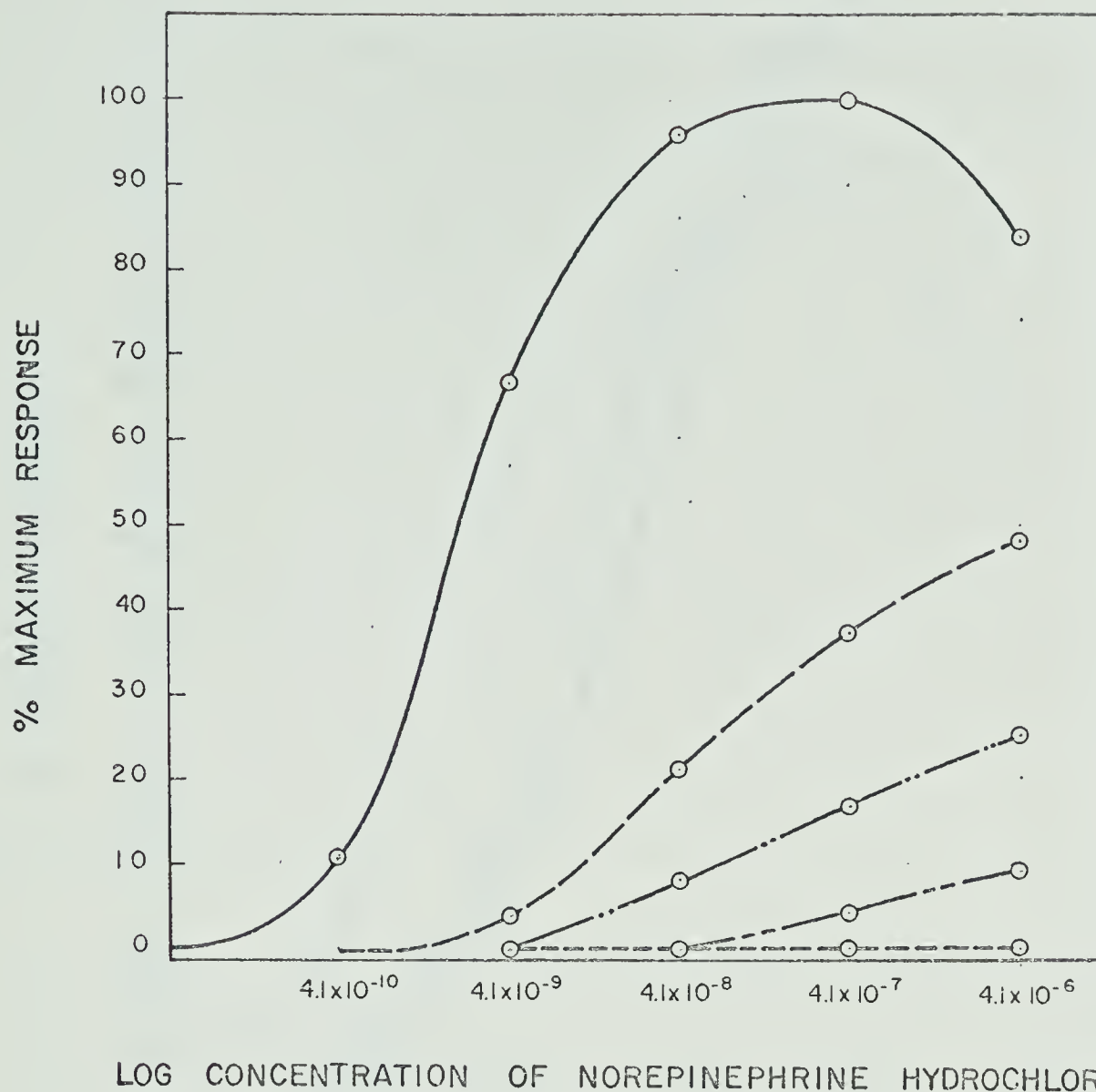
Experimental Strips	Control Strips
Strip 1 and 2	Strip 3 and 4
1. Incubate with dibenamine hydrochloride (1×10^{-6}) for 20 min	Incubate with dibenamine hydrochloride (1×10^{-6}) for 20 min
2. Wash once	Wash once

3. Test with epinephrine dose range	Test with epinephrine dose range
4. Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr
5. Incubate with propranolol (1.48×10^{-5}) for 10 min	Omit
6. Without changing bath fluid, test with epinephrine dose range	Test with epinephrine dose range
7. Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr
8. Test with epinephrine dose range	Test with epinephrine dose range
9. Wash at 15 min intervals for 1 hr	Wash at 15 min intervals for 1 hr
10. Test with epinephrine dose range	Test with epinephrine dose range

The experiment was repeated and the results are shown in Figs. 9A and 9B. This experiment was then repeated 7 times with ^{14}C -dibenamine hydrochloride in place of unlabeled dibenamine hydrochloride. The tissues were removed from the bath and the radioactivity of each of the strips was determined by the method previously described. The data is recorded in Figs. 10A and 10B and Table I.

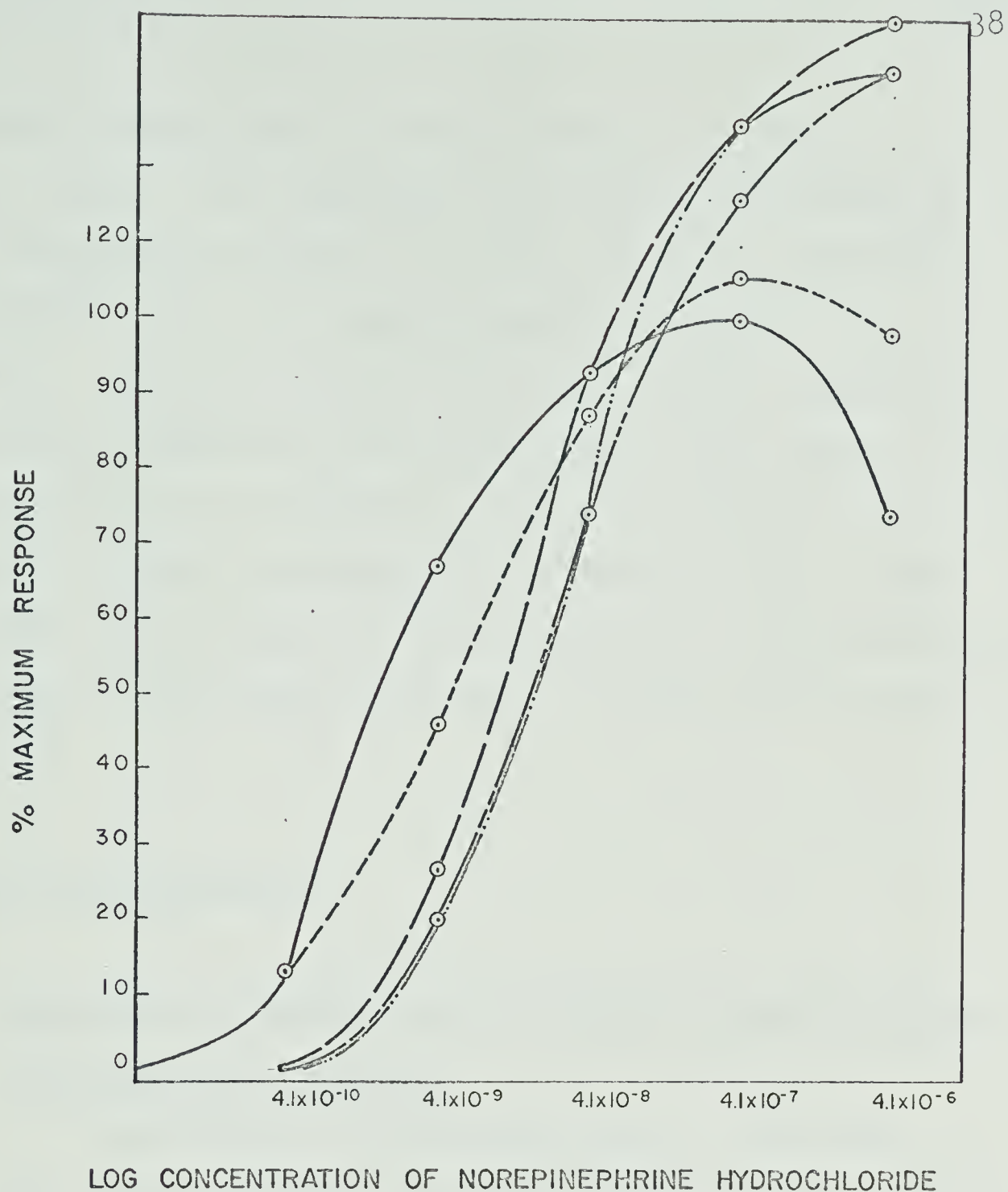
(d) The Effect of Washing ^{14}C -Dibenamine Labeled Rabbit Aortic Strips With Propranolol Solution.

The response of four aortic strips to epinephrine was recorded. The tissues were then washed at 15 min intervals until they relaxed. They were then exposed to ^{14}C -dibenamine hydrochloride (1×10^{-6}) for 20 min, the bath



LOG CONCENTRATION OF NOREPINEPHRINE HYDROCHLORIDE

Fig. 6A Effects of phenoxybenzamine and propranolol on the dose-response curve of norepinephrine in rabbit aortic strips. A control cumulative dose-response curve to norepinephrine was obtained, $\circ$ — $\circ$. All subsequent responses are compared to the maximum response obtained in the control dose-response curve. The dose-response curve, $\circ$ — $\circ$, was obtained immediately after incubation of the strip with phenoxybenzamine (1×10^{-7}) for 10 min. One hour later, the strip was incubated with propranolol (1.48×10^{-5}) for 10 min and the following curve, $\circ$ — $\circ$, obtained; 1 hr after propranolol, $\circ$ — $\circ$; $2\frac{1}{2}$ hrs $\circ$ — $\circ$ following propranolol.



LOG CONCENTRATION OF NOREPINEPHRINE HYDROCHLORIDE

Fig. 6B Effect of propranolol hydrochloride (1.48×10^{-5}) incubation for 10 min on the dose-response curve of norepinephrine in rabbit aortic strips. An initial cumulative dose-response curve to norepinephrine was obtained, $\circ-\circ$, and all subsequent responses are compared to the maximum contractile response obtained from this curve. The dose-response curve, $\circ-\cdots-\circ$, was obtained $1\frac{1}{2}$ hrs after the initial control curve (at the time when strips 1 and 2 were being tested for degree of blockade after phenoxylbenzamine incubation). 1 hr later, the strip was incubated with propranolol and immediately afterwards the following curve, $\circ-\cdots-\circ$, obtained; 1 hr after propranolol, $\circ-\cdots-\circ$; $2\frac{1}{2}$ hrs following propranolol, $\circ-\circ$.

solution drained, and one strip removed and stored in the frozen state. The remaining three strips were washed at 15 min intervals with McEwen solution containing propranolol (1.48×10^{-5}), for the following periods of time: strip 2, 1 hr; strip 3, 3 hrs; and strip 4 for 6 hrs. After washing, the strips were stored in the frozen state. As a control, rabbit aortic strips were treated in a similar manner, but with the omission of the propranolol in the washing fluid. The radioactivity of each of the two groups was then determined by a method previously discussed. The data is recorded in Table II.

Results and Discussion

(a) The Effect of Propranolol on Phenoxybenzamine-Induced α -Adrenergic Blockade

The results obtained with strip 1 are shown in Fig. 6A. It is apparent that after exposure of the strip to phenoxybenzamine hydrochloride (1×10^{-7}) for 10 min, complete α -adrenergic blockade takes place. After the strip had been incubated with propranolol (1.48×10^{-5}) for 10 min, the strip was found to become progressively more responsive to norepinephrine with time.

The results obtained with strip 3 are shown in Fig. 6B. The administration of propranolol (1.48×10^{-5}) for 10 min had an influence on the subsequent response of the rabbit aortic strip to norepinephrine. Following propranolol

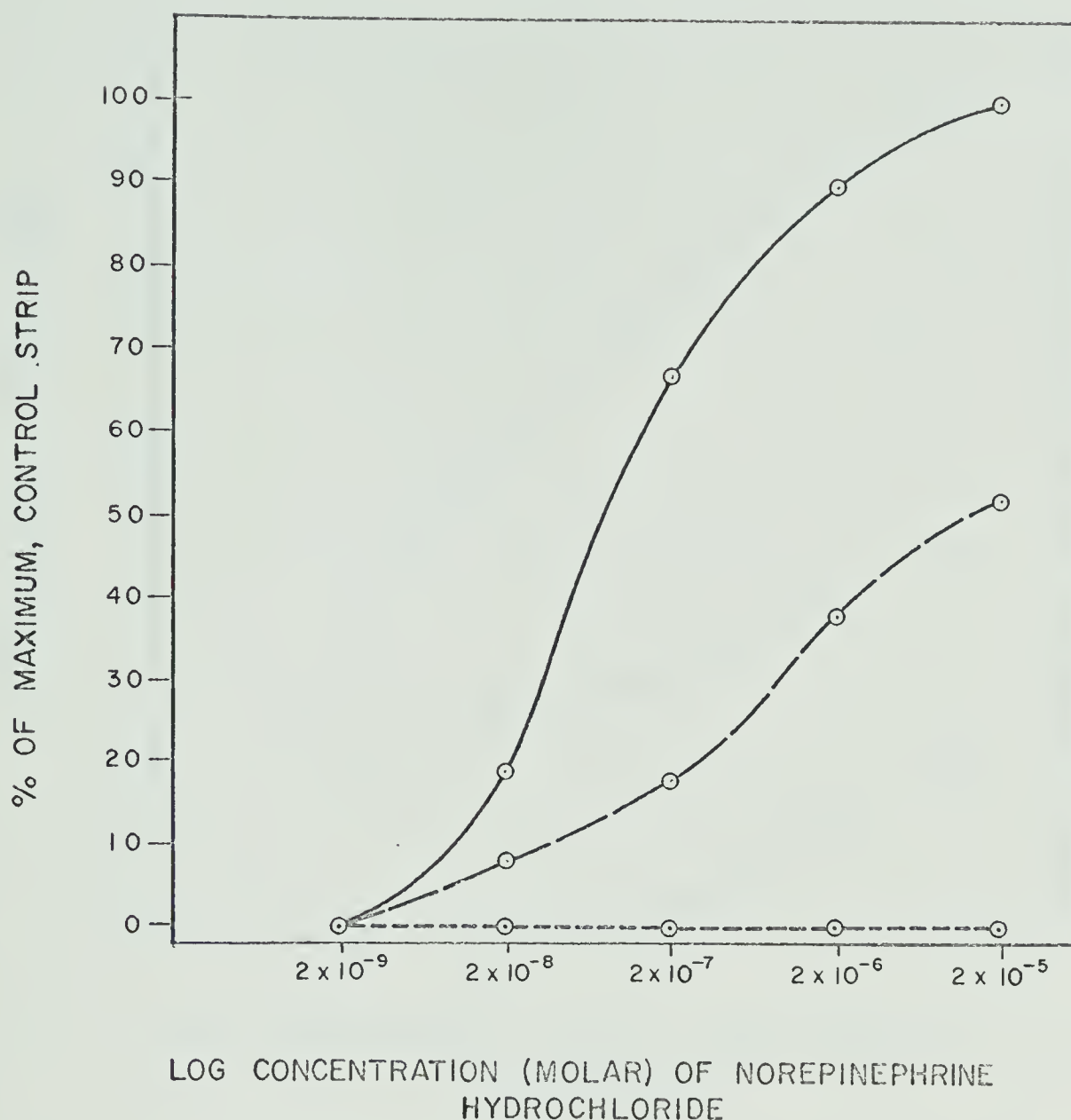


Fig. 6C Effects of phenoxybenzamine and propranolol on the dose-response curve of norepinephrine in isolated rabbit aortic strips. Two strips were treated in an identical fashion, except that the control strip was not treated with phenoxybenzamine. The control dose-response curve $\circ$ — $\circ$ was obtained $2\frac{1}{2}$ hrs after propranolol incubation. The response of experimental strips immediately after $3 \times 10^{-7} \text{M}$ ($1 \times 10^{-7} \text{g/ml}$) phenoxybenzamine (PBZ) $\circ$ --- $\circ$; and $2\frac{1}{2}$ hrs after the phenoxybenzamine treated strip had been incubated with propranolol ($5 \times 10^{-5} \text{M}$) $\circ$ — $\circ$. Treatment of experimental and control strips correspond to strips 1 and 3 (section 'a', experimental) in our studies.

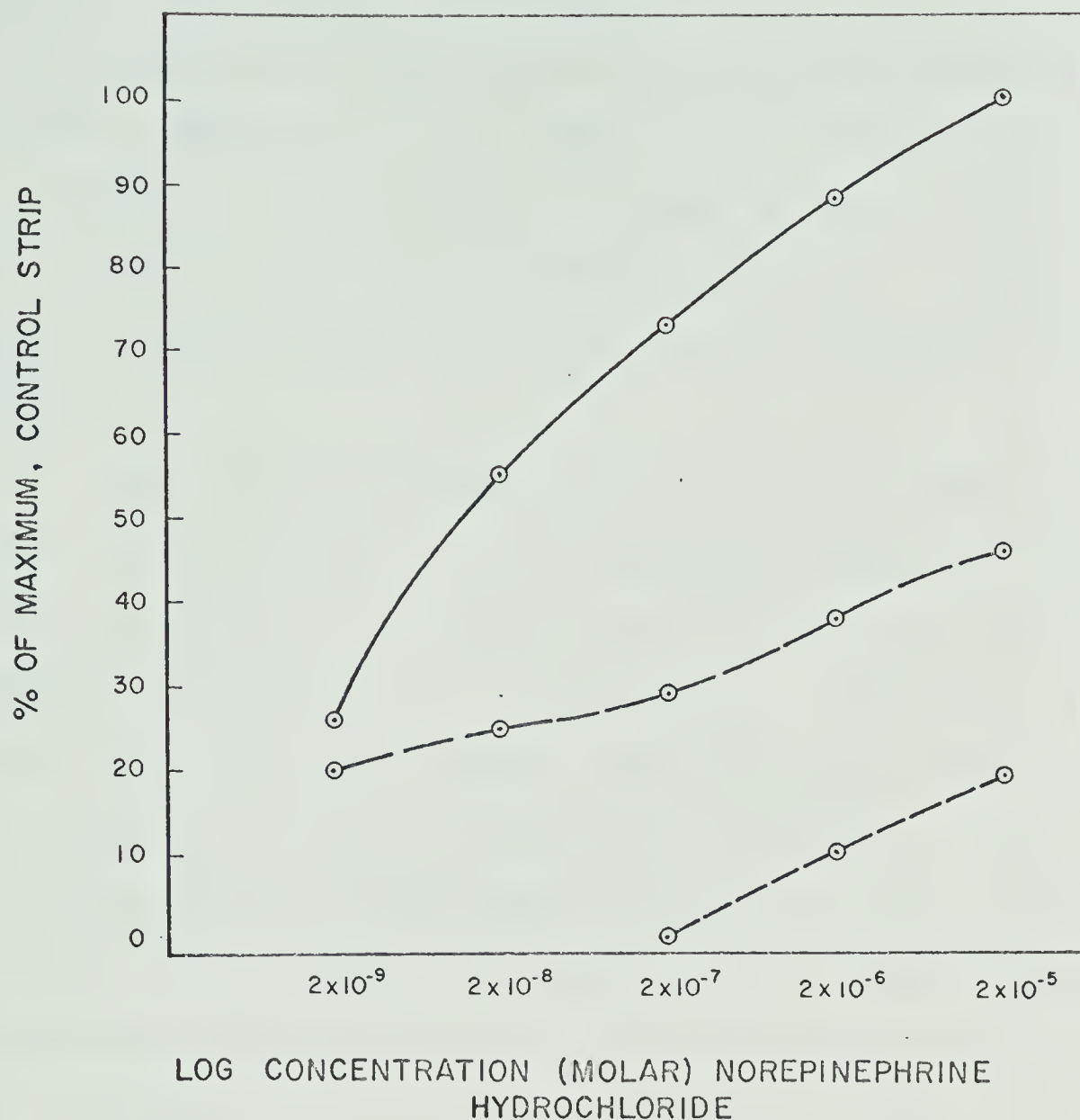


Fig. 6D Effects of phenoxybenzamine and propranolol in the dose-response curve of norepinephrine on the isolated rabbit aortic muscle preparation. Two strips were treated in identical fashion, except that the control strip was not treated with phenoxybenzamine, control preparation $\circ$ — $\circ$, experimental strip after 3×10^{-7} M phenoxybenzamine (PBZ), $\circ$ --- $\circ$; and after PBZ followed by propranolol, 5×10^{-5} M, $\circ$ — $\circ$. Treatment of the control strip and experimental strips correspond to treatment of strips 1 and 3 (section (a) experimental) in our studies(modified from Olivares et al., 1967).

administration, there is decreased sensitivity to low doses of norepinephrine and increased sensitivity to high doses. For direct comparison of our results with those of Olivares et al. (1967) recorded in Fig. 6D, some of the data from Figs. 6A and 6B have been replotted in Fig. 6C. Comparison of Figs. 6C and 6D show our results to be in fairly close agreement.

The results obtained with strip 2 are shown in Fig. 6E. After incubation with phenoxybenzamine hydrochloride (1×10^{-7}) for 10 min, the rabbit aortic strip was found to be completely unresponsive to norepinephrine. However, it is apparent (Fig. 6E) that washing the strip over a period of time results in recovery of partial sensitivity. This finding is in direct contrast with that of Olivares et al. (1967), who claimed that the blockade produced by phenoxybenzamine hydrochloride could not be overcome by the washing procedure alone. A comparison of the results shown in Figs. 6A and 6E reveals that the only difference produced by the propranolol treatment as compared to the normal washing procedure, was an increased sensitivity to the highest concentration of norepinephrine, viz., 4.1×10^{-6} . A comparison of the set of dose-response curves following propranolol treatment (Fig. 6B), with the set of dose-response curves in which propranolol is omitted (Fig. 6F), shows a considerable difference. This is in disagreement with Olivares et al. (1967), who claimed that incubation of rabbit aortic strips with propranolol did not

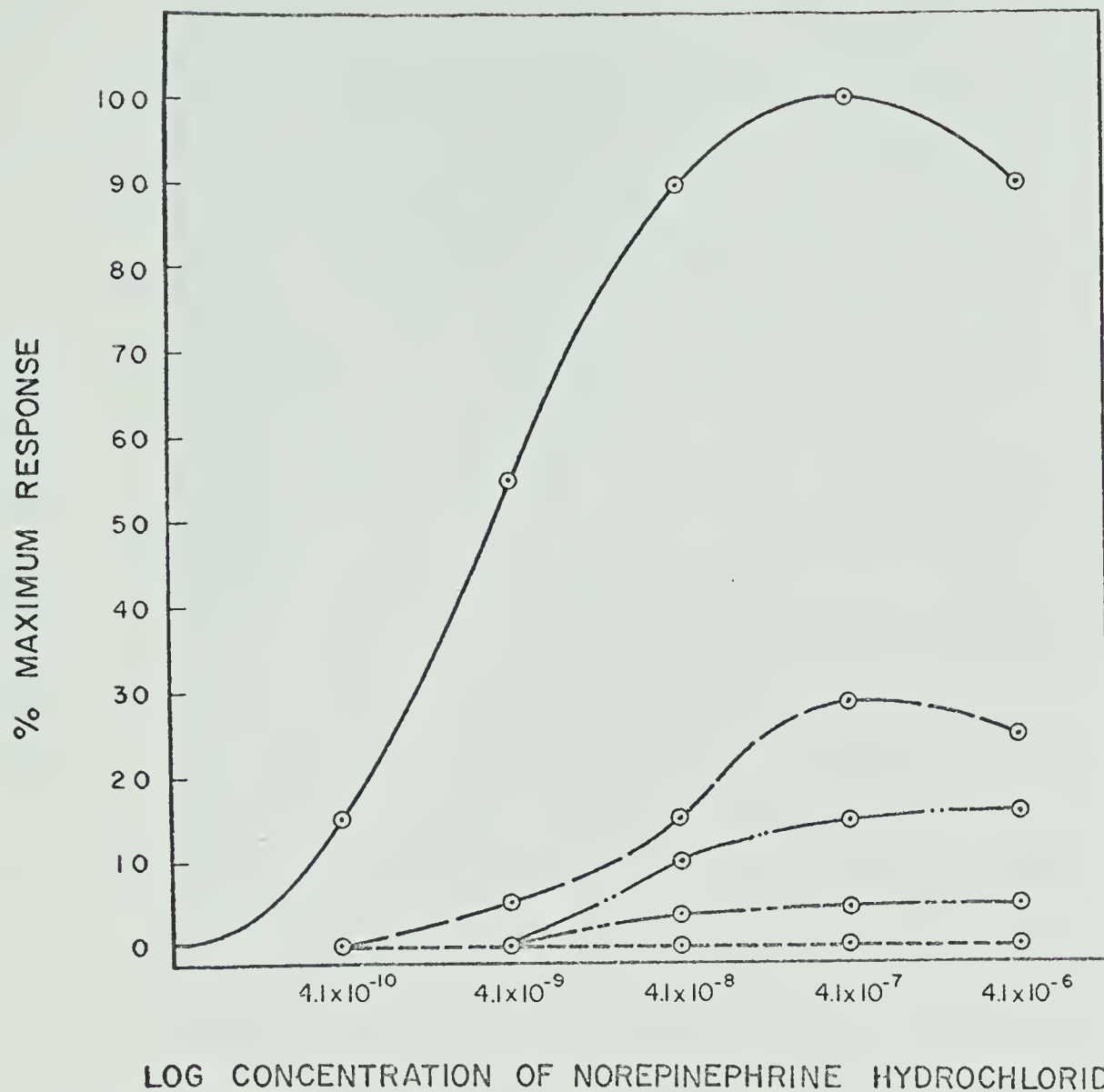
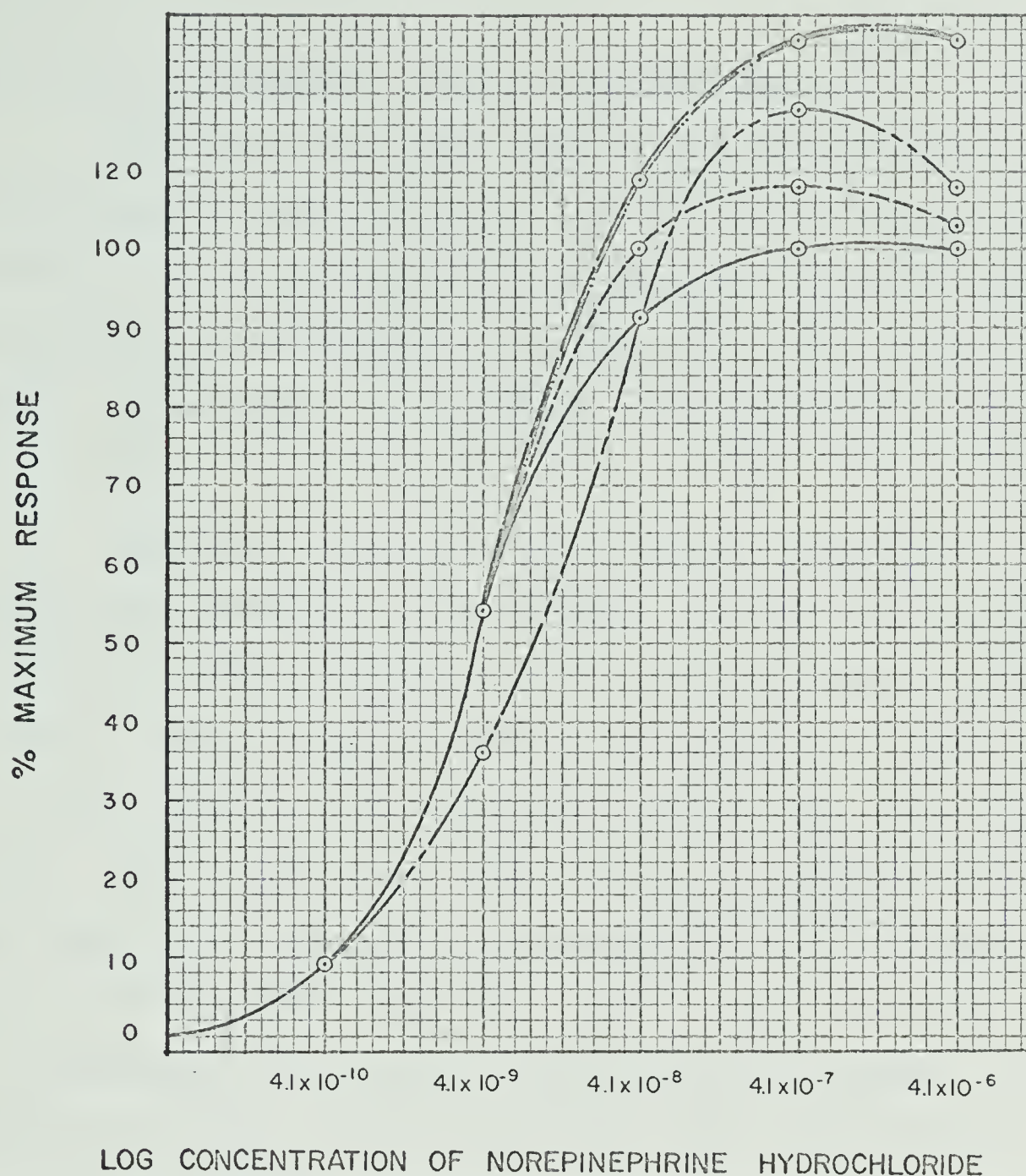


Fig. 6E Effect of washing on the response to norepinephrine in isolated rabbit aortic strips which have been incubated with phenoxybenzamine (1×10^{-7}). An initial dose-response curve to cumulative doses of norepinephrine, $\circ$ — $\circ$, was obtained. 1 hr later, the strip was incubated with phenoxybenzamine (1×10^{-7}) for 10 min, and subsequently subjected to the dose range of norepinephrine, $\circ$ --- $\circ$. After washing every 15 min with McEwen solution for 1 hr, the following dose-response curve to cumulative doses of norepinephrine were obtained $\circ$ --- $\circ$; 2 hrs later $\circ$ — $\circ$; $3\frac{1}{2}$ hrs later $\circ$ — $\circ$.



alter the dose-response curve to norepinephrine.

In view of the fact that strips treated with phenoxybenzamine hydrochloride (1×10^{-7}) for 10 min, regained sensitivity to norepinephrine upon repeated washings (Fig. 6E) we considered it of interest to repeat the above study using a higher concentration of phenoxybenzamine hydrochloride viz., 1×10^{-6} .

The results obtained with strip 1 (Fig. 7A) indicated that complete α -adrenergic blockade was produced by incubation with phenoxybenzamine hydrochloride (1×10^{-6}) for 10 min. After incubation with propranolol (1.48×10^{-5}) for 10 min, complete α -adrenergic blockade was found to persist. Over a period of time, the aortic strip regained a modest degree of sensitivity to norepinephrine at higher doses of the agonist. From Fig. 7B, it can be observed that the α -adrenergic blockade was unaffected by washing. Comparison of Figs. 7A and 7B reveals that propranolol treatment does affect sensitivity to norepinephrine after α -adrenergic blockade. Control strips 3 and 4 in this experiment, behaved similarly to control strips 3 and 4 (Figs. 6B and 6F) in the previous experiment.

(b) The Effect of Propranolol on Dibenamine-Induced
 α -Adrenergic Blockade

When rabbit aortic strips are incubated with dibenamine hydrochloride (3×10^{-6}) for 20 min, complete

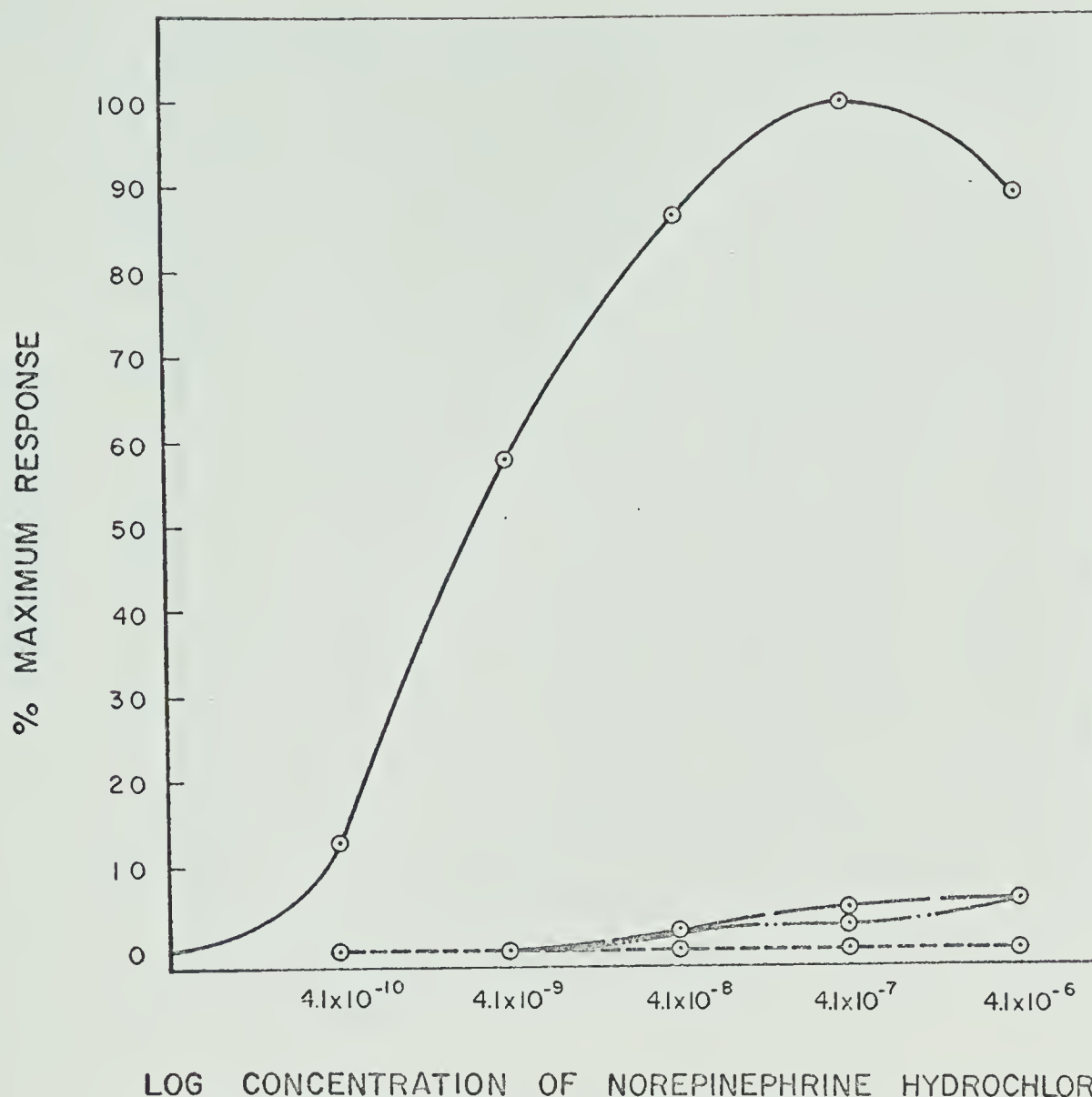
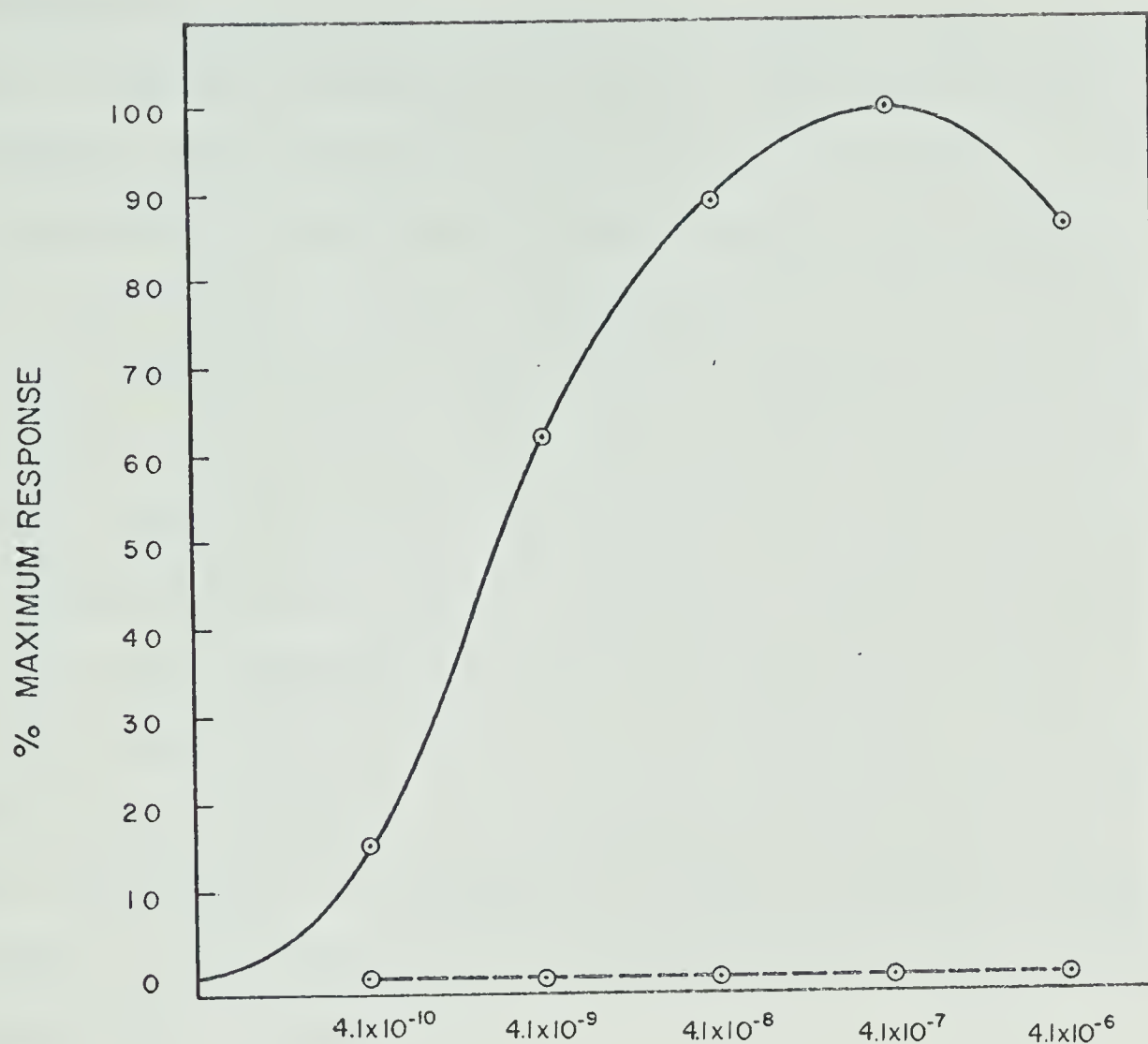


Fig. 7A Effect of phenoxybenzamine (1×10^{-6}) and propranolol (1.48×10^{-5}) on the dose-response curve of norepinephrine in rabbit aortic strips. A cumulative dose-response curve to norepinephrine was obtained, $\circ$ — $\circ$. All subsequent responses are compared to the maximum response obtained in the control response in the control dose-response curve. The symbol $\circ$ --- $\circ$ represents the response of the strip to norepinephrine immediately after phenoxybenzamine (1×10^{-6}) incubation. After propranolol (1.48×10^{-5}) incubation, the response is represented by, $\circ$ ----- $\circ$. The response to norepinephrine 1 hr after propranolol incubation is represented thus, $\circ$ —•— $\circ$; $2\frac{1}{2}$ hrs after propranolol incubation, $\circ$ — $\circ$.



LOG CONCENTRATION OF NOREPINEPHRINE HYDROCHLORIDE

Fig. 7B Effect of washing on the response to norepinephrine in isolated rabbit aortic strip which has been incubated with phenoxybenzamine (1×10^{-6}) for 10 min. An initial dose-response curve to cumulative doses of norepinephrine, $\circ$ — $\circ$, was obtained. 1 hr later, the strip was incubated with phenoxybenzamine (1×10^{-6}) for 10 min and then exposed to the dose range of norepinephrine. After washing every 15 min with McEwen solution the strip was tested with cumulative doses of norepinephrine 1 hr, 2 hrs, and $3\frac{1}{2}$ hrs later, $\circ$ — $\circ$; total α -adrenergic blockade was demonstrated over the entire period of time.

α -adrenergic blockade persists for longer than 3 hrs (Yong, 1968). Results obtained from strip 1 (Fig. 8A) showed that incubation with dibenamine hydrochloride (3×10^{-6}) for 20 min had completely eliminated responsiveness to epinephrine. After exposing the strip to propranolol (1.48×10^{-5}) for 10 min, the sensitivity of the strip to epinephrine increased with time. With strip 2 (Fig. 8B) in which propranolol treatment was omitted, the strip did not regain sensitivity to epinephrine after repeated washing. Control strips 3 and 4 in this experiment behaved similarly to control strips 3 and 4 (Figs. 6B and 6F) in a previous experiment. These results indicate that propranolol treatment does effect sensitivity to epinephrine after dibenamine hydrochloride blockade in the same manner as observed after phenoxybenzamine hydrochloride blockade.

(c) The Effect of Propranolol on ^{14}C -Dibenamine-Induced α -Adrenergic Blockade

In these groups of experiments, we used ^{14}C -dibenamine hydrochloride at a concentration of 1×10^{-6} because this is the minimal dose of the blocking agent required to cause total α -adrenergic blockade (Yong, 1968). If, in fact, dibenamine hydrochloride is displaced from the α -adrenergic receptors, as suggested by Olivares et al. (1967), it was felt that with this minimal dose, any changes in occupation of the receptors would be best observed.

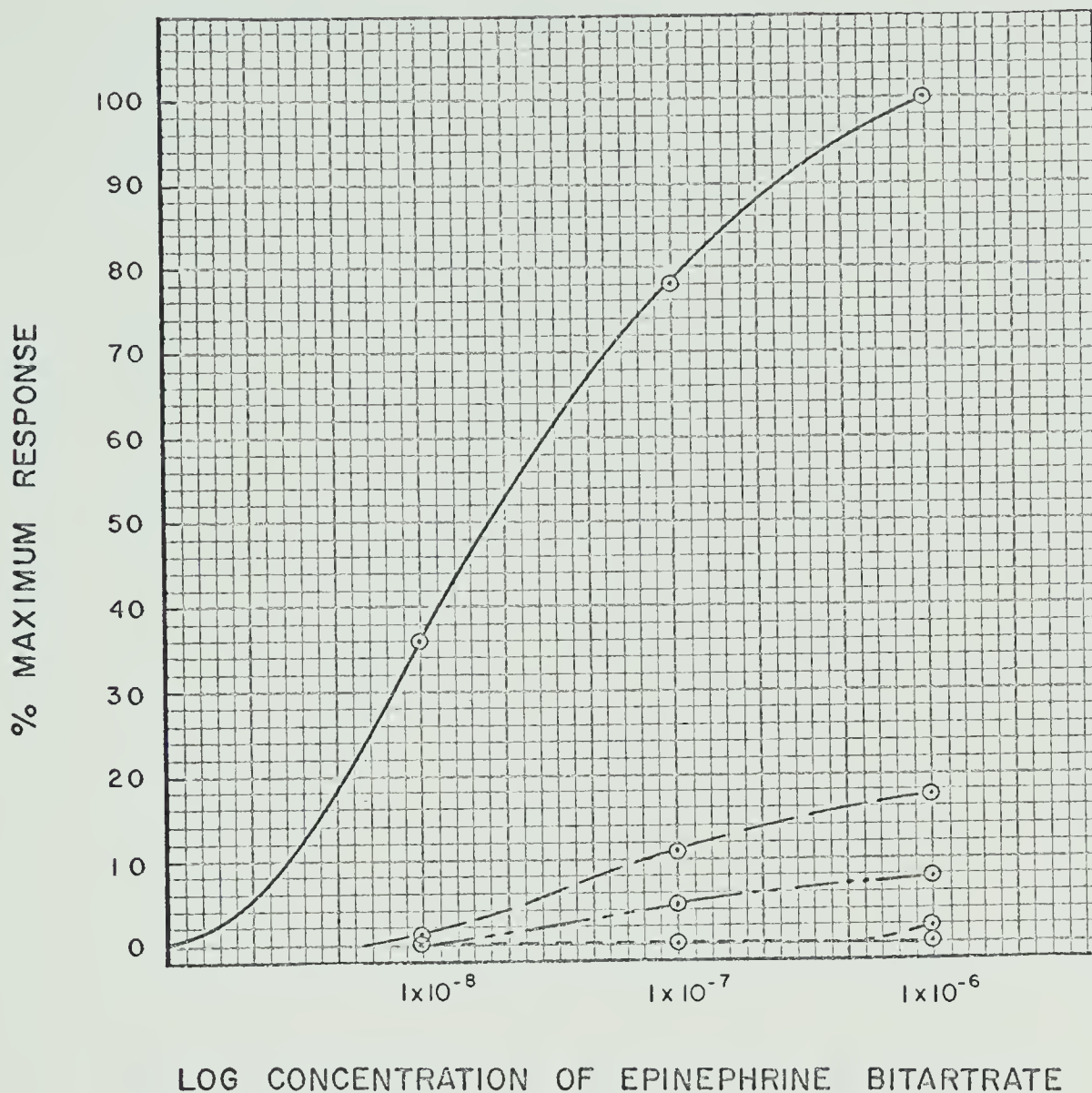


Fig. 8A Effects of dibenamine (3×10^{-6}) and propranolol (1.48×10^{-5}) on the dose-response curve to epinephrine in rabbit aortic strips. A cumulative dose-response curve, $\circ$ — $\circ$, to epinephrine was obtained. All subsequent responses are compared to the maximum response of the control curve. The strips were incubated with dibenamine (3×10^{-6}) for 20 min and then exposed to epinephrine, $\circ$ ----- $\circ$. 1 hr later, the strip was exposed to propranolol (1.48×10^{-5}) for 10 min and immediately thereafter exposed to the dose range of epinephrine and the following curve obtained $\circ$ --- $\circ$; 1 hr after propranolol $\circ$ —...— $\circ$; $2\frac{1}{2}$ hrs following propranolol incubation $\circ$ — $\circ$. Each point represents the mean of 3 experiments.

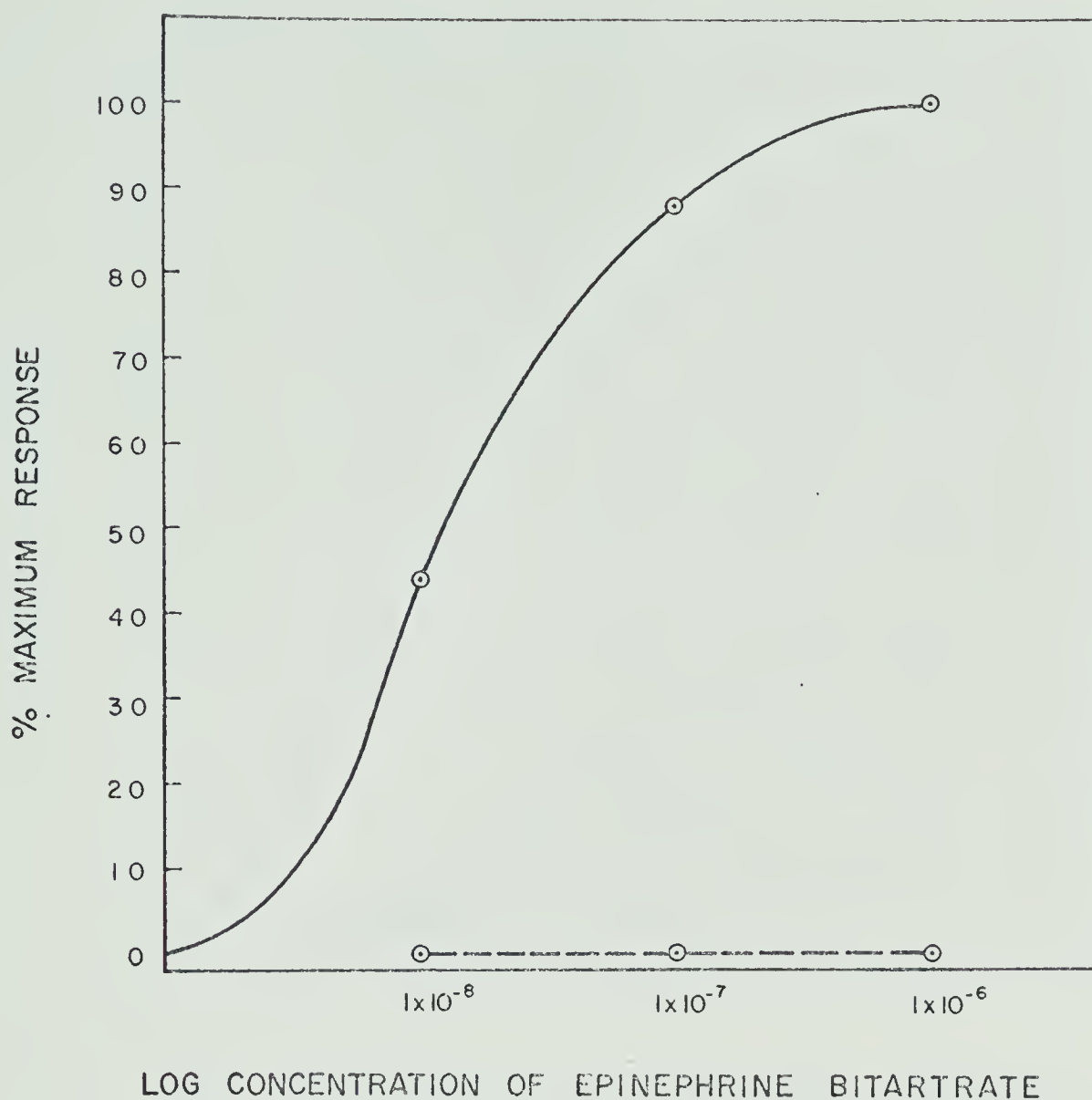


Fig. 8B Effect of washing over a period of time on the dose-response curve to epinephrine in rabbit aortic strips which have been incubated with dibenamine (3×10^{-6}). An initial dose-response curve $\circ$ — $\circ$, was obtained from cumulative doses of epinephrine. 1 hr later, the strip was incubated with dibenamine (3×10^{-6}) for 20 min. After washing the tissue every 15 min, the strips were exposed to cumulative doses of epinephrine 1 hr, 2 hrs, and $3\frac{1}{2}$ hrs later, $\circ$ --- $\circ$; total α -adrenergic blockade was observed over the entire time period.

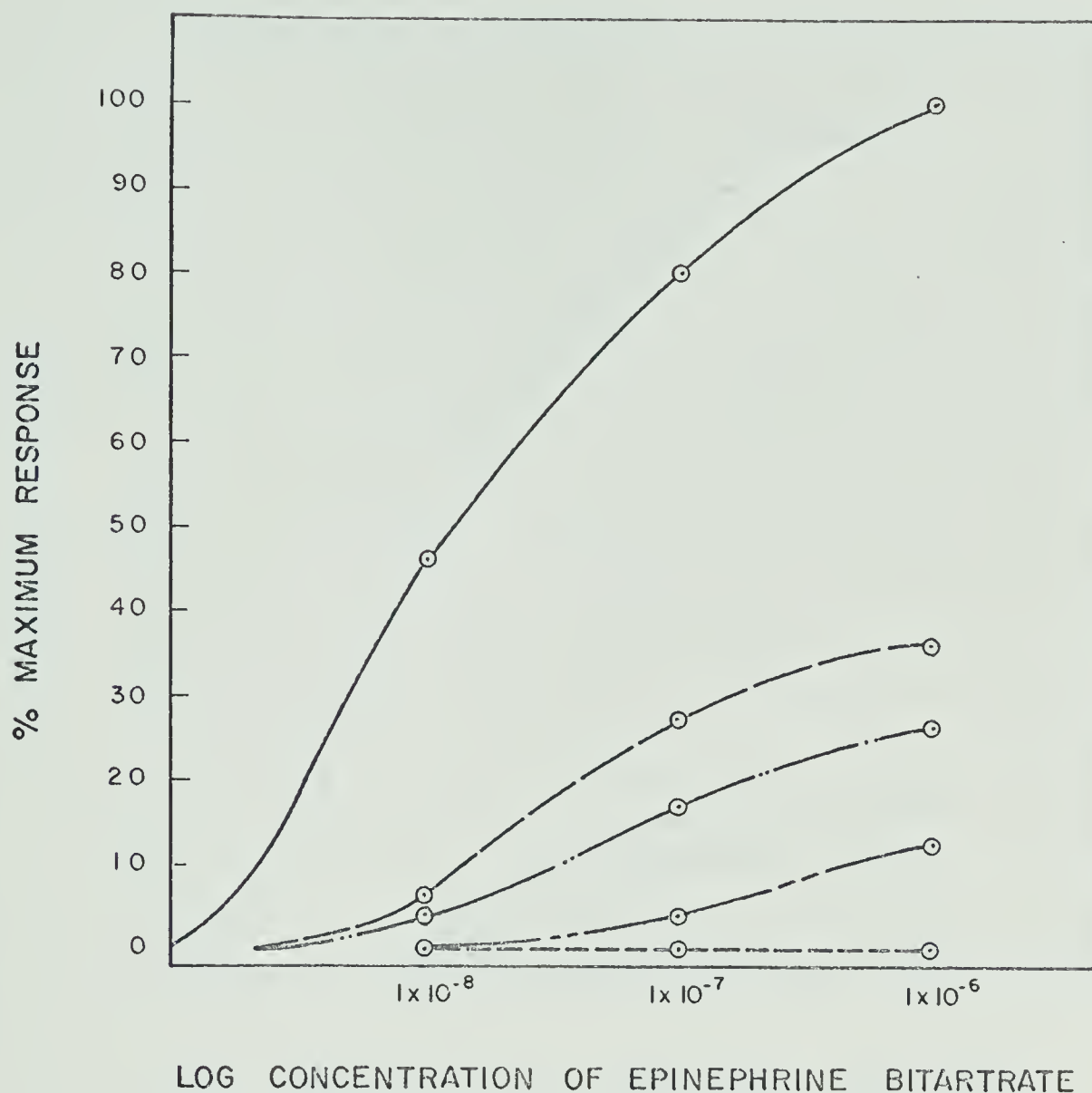


Fig. 9A Effect of propranolol on dibenamine-induced α -adrenergic blockade in rabbit aortic strips. A control cumulative dose-response curve to epinephrine was obtained, $\circ$ — $\circ$. All subsequent responses are compared to the maximum response obtained in this curve. After incubation of isolated rabbit aortic strips with dibenamine (1×10^{-6}) for 20 min, the response to the dose range of epinephrine, $\circ$ ---- $\circ$, was recorded. 1 hr later, the strips were incubated with propranolol (1.48×10^{-5}) for 10 min and the subsequent response to epinephrine measured, $\circ$ — $\circ$; 1 hr after propranolol $\circ$... $\circ$; 2 $\frac{1}{2}$ hrs following propranolol incubation $\circ$ — $\circ$. Each point represents the mean of 4 values.

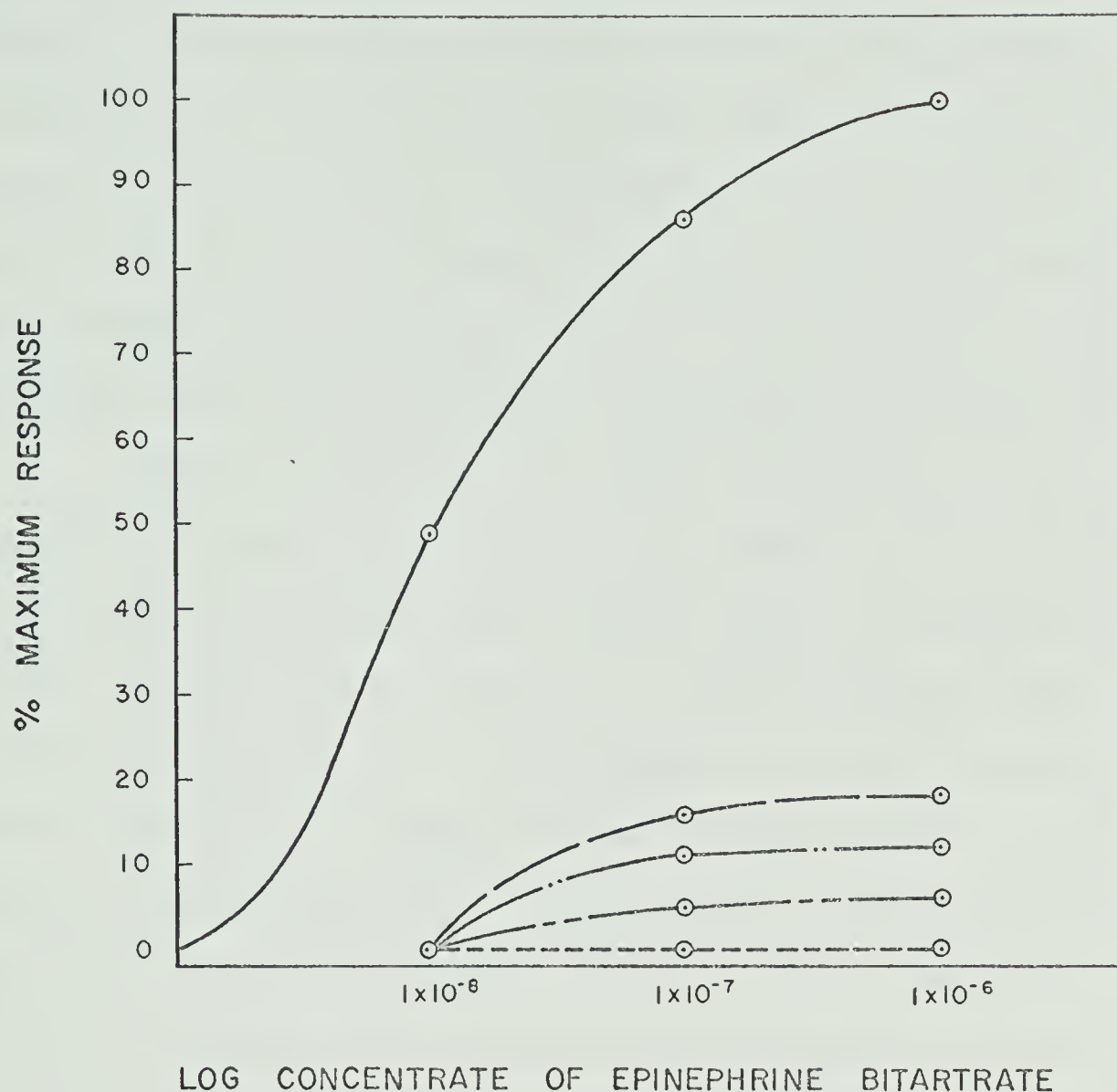


Fig. 9B Effect of washing over a period of time on dibenamine-induced α -adrenergic blockade in isolated rabbit aortic strips. A control cumulative dose-response curve to epinephrine, $\bigcirc$ — $\bigcirc$, was obtained. Again all subsequent responses will be compared with the maximal response of this initial curve. $\bigcirc$ — $\bigcirc$ represents the response to epinephrine, immediately after rabbit aortic strips had been incubated in dibenamine (1×10^{-6}) for 20 min; $\bigcirc$ — $\bigcirc$ is the response 1 hr later; $\bigcirc$ — $\bigcirc$, 2 hrs later; and $\bigcirc$ — $\bigcirc$ $3\frac{1}{2}$ hrs after the dibenamine blockade. Each point represents a mean of 4 values.

The results shown in Figs. 9A and 9B, using unlabeled dibenamine hydrochloride (1×10^{-6}) for 20 min, show that propranolol treated strips show a greater degree of recovery of sensitivity to epinephrine with time (Fig. 9A) than did the control tissues (Fig. 9B). Thus, the results with dibenamine hydrochloride (1×10^{-6}) are similar to those previously found with dibenamine hydrochloride (3×10^{-6}).

These experiments were then repeated several times using ^{14}C -dibenamine hydrochloride in place of the unlabeled α -adrenergic blocking agent and the results obtained shown in Figs. 10A and 10B and Table I. It is apparent that the recovery of sensitivity to epinephrine is significantly greater ($P < 0.05$) with time in experimental strips than with control strips (Table I). If the ideas of Olivares et al. (1967) are correct one should expect to find less radioactivity in experimental aortic strips than in control strips. The radioactivity obtained in our experimental strips was 98 ± 19 dpm per mg dry weight, while that of the control strips was 97 ± 26 dpm per mg dry weight of aortic tissue (Table I). This data provides no support for the proposal of Olivares et al. (1967).

(d) The Effect of Washing ^{14}C -Dibenamine Treated Aortic Strips With Propranolol Solution

From the results in the previous section, it appeared that a single dose of propranolol had no effect on the amount of dibenamine hydrochloride bound to the rabbit

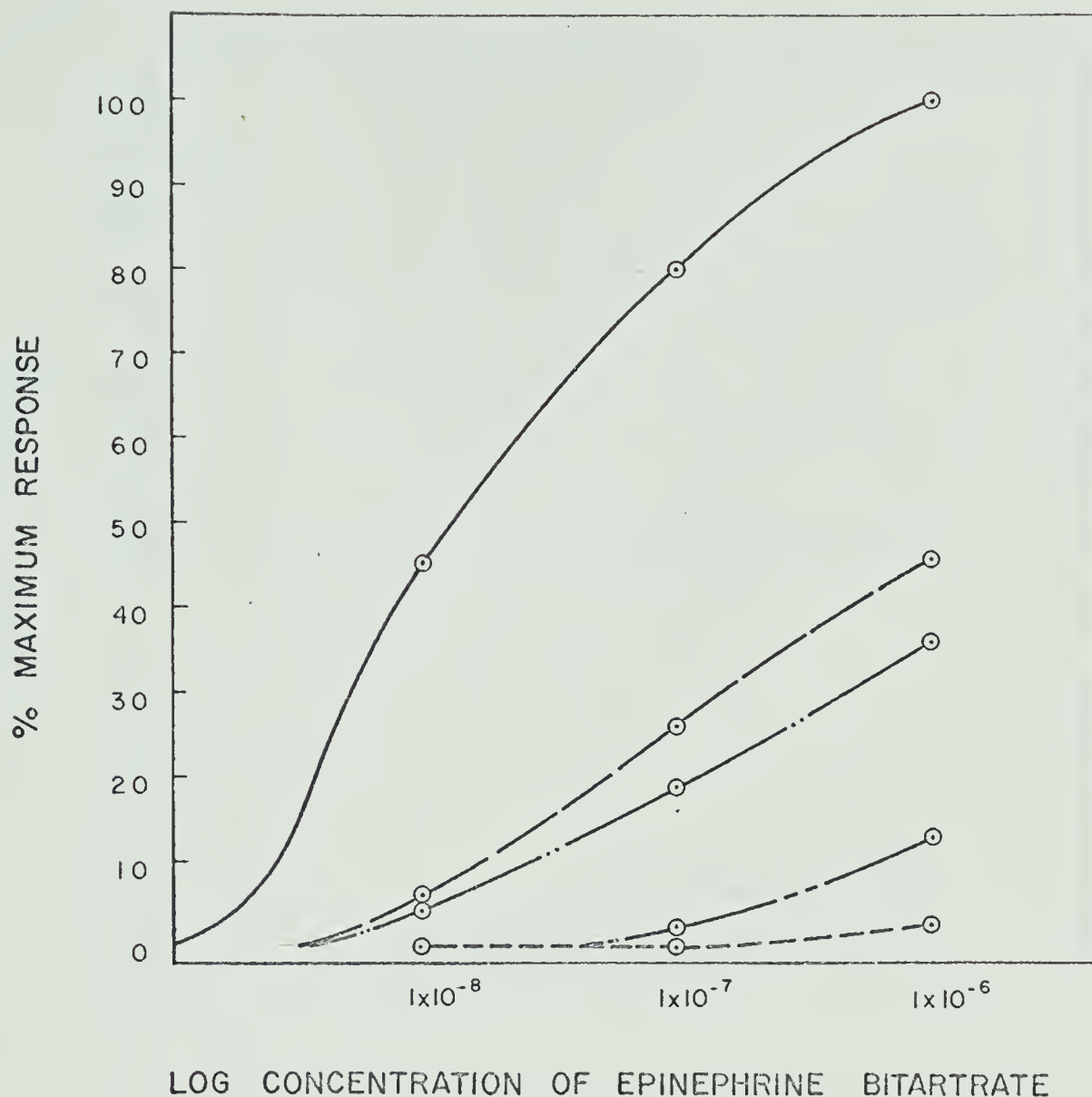


Fig. 10A Effect of ^{14}C -dibenamine (1×10^{-6}) and propranolol (1.48×10^{-5}) on the dose-response curve of epinephrine in rabbit aortic strips. A control cumulative dose-response curve to epinephrine was obtained, $\circ$ — $\circ$. All subsequent responses are compared to the maximum response obtained in this curve. After incubation of the aortic strips with ^{14}C -dibenamine (1×10^{-6}) for 20 min, the response to the dose range of epinephrine, $\circ$ --- $\circ$, was recorded. 1 hr later, the strips were incubated with propranolol (1.48×10^{-5}) for 10 min and the subsequent response to epinephrine measured, $\circ$ --- $\circ$; 1 hr after propranolol, $\circ$ — $\circ$; $2\frac{1}{2}$ hrs following propranolol incubation, $\circ$ — $\circ$. The values plotted are the means of 14 rabbit aortic strips.

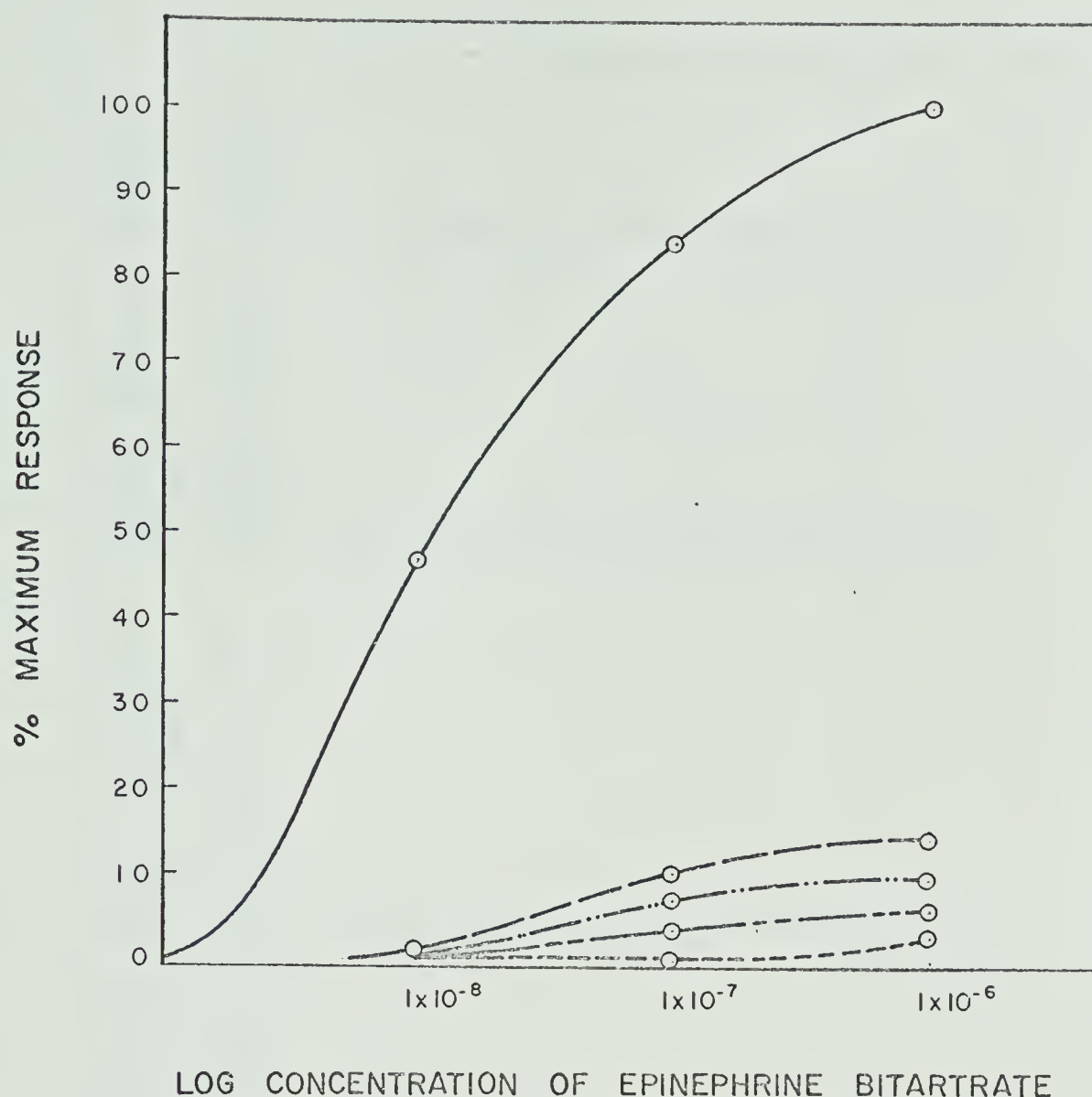


Fig. 10B Effect of washing over a period of time on ^{14}C -dibenamine-induced α -adrenergic blockade in isolated rabbit aortic strips. A control cumulative dose-response curve, $\circ$ — $\circ$, to epinephrine was obtained. As done previously, all subsequent responses were compared to the maximal response of their initial curve. $\circ$ --- $\circ$ represents the response to epinephrine, immediately after incubation of aortic strips with ^{14}C -dibenamine (1×10^{-6}) for 20 min; $\circ$ --- $\circ$ is the response 1 hr later; $\circ$ — $\circ$, 2 hrs later; and $\circ$ — $\circ$, $3\frac{1}{2}$ hrs after the dibenamine blockade. All points plotted represent the mean of 14 rabbit aortic strips.

TABLE I THE EFFECT OF PROPRANOLOL ON THE DEGREE OF BINDING OF ^{14}C -DIBENAMINE HYDROCHLORIDE AND THE RECOVERY OF SENSITIVITY TO EPINEPHRINE IN ^{14}C -DIBENAMINE HYDROCHLORIDE TREATED RABBIT AORTIC STRIPS.

Dry Weight of Aortic Strips (mg)		*Percent of Original Response to Epinephrine after final Washout		Total dpm per mg Dry Weight	
Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip
5.8	8.9	19	6	86	99
8.3	5.7	17	10	107	100
7.9	8.7	41	21	92	98
9.2	8.4	42	33	96	93
6.5	7.0	34	0	131	155
8.1	10.5	44	0	140	115
10.0	9.9	30	4	71	77
10.2	9.4	29	0	78	76
8.4	10.6	27	25	115	86
10.7	11.0	36	11	85	101
7.4	7.8	33	8	98	83
8.4	9.1	16	7	101	100
9.9	8.1	33	12	97	80
8.9	9.5	32	10	69	157
Mean 8.6	8.9	30	11	98	97
S.D. ± 1.4	± 1.5	± 8.7	± 9.7	± 19	± 26
P Value		<0.05		>0.05	

* This was calculated as described in 'Experimental' section in Chapter II for each dose of epinephrine used. The values represent the mean of the response from 3 doses.

aortic strips. It was therefore decided to see if washing with McEwen solution containing propranolol (1.48×10^{-5}) had any effect on the retention of ^{14}C -dibenamine hydrochloride by rabbit aortic strips.

Upon examination of the results in Table II, it is apparent that washing ^{14}C -dibenamine treated rabbit aortic strips with propranolol containing solution every 15 min for a period up to 6 hrs, had no significant effect on the amount of ^{14}C -dibenamine hydrochloride retained by the strips. The data in this experiment, like that in the previous experiment, do not provide support for the hypothesis of Olivares et al. (1967). Thus, on the basis of this data, it is unlikely that α -adrenergic blocking agents of the β -haloalkylamine type are displaced from the receptor sites by propranolol. However, the results from these labeling studies are not conclusive for the following reasons: The work of Yong and Marks (1969) indicated that when the α -adrenergic receptors of rabbit aortic strips are blocked by ^{14}C -dibenamine hydrochloride (1×10^{-6}) only a small proportion of the labeled drug in the strips is bound to the α -adrenergic receptors. The remainder is bound to the other receptors and non-specific sites. Thus, it is possible that a small proportion of the ^{14}C -dibenamine hydrochloride bound to the α -adrenergic receptors might have been removed and that our methods might not have been sensitive enough to detect the removal. However, it is likely that if propranolol possessed the ability to displace dibenamine

TABLE II EFFECT OF WASHING ^{14}C -DIBENAMINE LABELED
RABBIT AORTIC STRIPS WITH PROPRANOLOL SOLUTION

Aortic Strips	Total dpm per mg dry weight of strip $\pm$ S.D.			
	0 hr	1 hr	3 hr	4 hr
Wash* with Propranolol (1.48×10^{-5}) in McEwen Solution	$\dagger$ 141 ± 29	129 ± 55	80 ± 16	94 ± 20
Wash with McEwen Solution	132 ± 18	110 ± 36	97 ± 10	90 ± 10
P Value	>0.05	>0.05	>0.05	>0.05

* Washing was carried out at 15 min intervals.

$\dagger$ Each value is the mean of 3 aortic strips.

hydrochloride from the α -adrenergic receptors it would also have the ability to displace it from other receptors and sites. Since this was not observed, it appears unlikely that α -adrenergic blocking agents are displaced from receptor sites by propranolol. Thus, our results indicate that the second hypothesis of Hull et al. (1960) is correct viz., that the β -adrenergic blocking agent, by combining with the β -receptors, allows the revealing of those α -receptor sites which have escaped blockade by the α -adrenergic blocking agent.

General Discussion

The following additional evidence offers support to the second hypothesis of Hull et al. (1960): (1) From Figs. 6B and 6F, it is obvious that propranolol treatment of control isolated rabbit aortic strips enhances the response of the strip to agonists at higher doses. This observation, which is contrary to that of Olivares et al. (1967), indicates that β -receptors which subserve relaxation are present in these strips. (2) A comparison of the degree of recovery that occurred when different concentrations of phenoxybenzamine hydrochloride were used to treat rabbit aortic strips prior to propranolol incubation shows that as the concentration of phenoxybenzamine hydrochloride was increased, the degree of apparent recovery due to propranolol incubation was reduced (Figs. 6A and 7A). Similar findings were obtained with dibenamine hydrochloride (Figs. 10A and 8A). This strongly

suggests that the larger dose of α -adrenergic blocking agent is more effective in reducing the number of free

α -receptors. Consequently, after blockade of the β -receptors with propranolol, the number of free α -receptors available for combination with epinephrine or norepinephrine is less and the response of the tissues is reduced. If the hypothesis of Olivares et al. (1967) was correct, no difference in recovery of response would be expected by increasing the dose of α -adrenergic blocking agent above that required to effect complete α -adrenergic blockade. (3) Another argument that Olivares et al. (1967) used to support their hypothesis was that propranolol treatment prior to phenoxybenzamine hydrochloride administration interfered with the establishment of α -adrenergic blockade. Yamamura and Horita (1968) found that the blood pressure in cats actually increased in response to epinephrine after cats were treated with propranolol (0.5mg/kg) and phenoxybenzamine hydrochloride (10mg/kg). However, they found that, as the β -adrenergic blockade wore off, a depressor response was observed instead of the initial pressor response, showing that phenoxybenzamine hydrochloride was still bound to the receptors and had not been displaced by propranolol. Yamamura and Horita (1968) also found that the pressor response to epinephrine could be regained by the administration of propranolol (0.5mg/kg). (4) This reappearance of the depressor response once the propranolol effect had disappeared is in direct contrast with the findings of Olivares et al. (1967), who found that the

recovery from α -adrenergic blockade persisted even after the propranolol (β -adrenergic blockade) had dissipated.

(5) Yamamura and Horita studied the effect of catecholamines on the blood pressure of the cat and showed that the α -adrenergic responses of agents possessing both α - and β -adrenergic properties were antagonized by phenoxybenzamine hydrochloride and exhibited partial recovery after subsequent administrations of propranolol. However, the α -adrenergic responses of agents possessing only an α -adrenergic stimulatory property (methoxamine and oxymetazoline) were not restored upon subsequent administration of propranolol. (6) The responses of the nictitating membrane, which contains primarily α -adrenergic receptors, to the various sympathomimetic amines were not restored to any significant extent after combined α - and β -adrenergic blockade.

As a result of their studies, Yamamura and Horita (1968) arrived at a similar conclusion to ours regarding the effect of propranolol on α -adrenergic blockade viz., the β -adrenergic blocking agent, by combining with the β -adrenergic receptor, reveals those α -adrenergic receptor sites which have escaped blockade by the α -adrenergic blocking agent. The second hypothesis of Hull et al. (1960) is also supported by Gonçalves and Osswald (1965) and Garrett et al. (1966). Both of these groups of workers used phenoxybenzamine hydrochloride as an α -adrenergic blocking agent and pronethalol as a β -adrenergic blocking agent.

It is surprising that Olivares et al. (1967) came

to the conclusion that propranolol displaced the α -adrenergic blocking agent, especially phenoxybenzamine hydrochloride, from the receptor, thus causing regeneration of sensitivity of the α -adrenergic receptors. Upon close examination of their results, in both their dog experiments and isolated rabbit aortic strips, it is apparent that in neither test systems did they ever demonstrate complete α -adrenergic blockade over the entire dose range of norepinephrine that they used. Thus, a relatively small unblocked group of α -adrenergic receptors would be able to express their pharmacological effects once the opposing effect of the β -adrenergic receptors has been removed by propranolol blockade.

CHAPTER IV THE RELATIONSHIP BETWEEN RECEPTOR PROTECTION
AND ^{14}C -DIBENAMINE HYDROCHLORIDE UPTAKE IN
ISOLATED RABBIT AORTIC STRIPS

Introduction

In order to gain a better understanding of drug-receptor interaction with the goal of labeling, and finally isolating drug receptors, many workers have followed the suggestions of Gaddum (1962), which were based on the receptor protection experiments of Furchgott (1954). A number of workers (Dikstein & Sulman, 1965; Yong & Marks, 1966; Lewis & Miller, 1966; Takagi et al., 1965; May et al., 1967) treated smooth muscle preparations with an agonist or short acting reversible antagonist in order to protect drug receptors from combination with a radioactively labeled irreversible antagonist. The amount of labeled drug taken up by the smooth muscle preparations was compared with that taken up by control preparations treated in a similar manner but with the omission of the receptor protecting agent. It was assumed that the difference in radioactivity between protected and unprotected strips represented that portion of the ^{14}C -labeled dibenamine hydrochloride combined with sites which might be in large part α -adrenergic receptor sites.

Waud (1962) pointed out that the concentration of the agonist used to protect the receptor was usually so large that an appreciable occupancy of sites other than receptors could result. Furchgott and Kirpekar (1963) demonstrated that epinephrine could prevent combination of dibenamine hydrochloride with the uptake sites of nerve terminals. Recently Moran et al. (1967) have pointed out

that agonists such as norepinephrine used in α -adrenergic protection studies possess substantial affinity for sites other than the α -adrenergic receptor. They have stressed the possibility that the non-specific binding sites in tissue may be much larger in number than the α -adrenergic receptor sites themselves. Hence, if receptor protection is to be used as a tool in labeling studies, it becomes vital to know whether there exists any relationship between receptor protection and the count difference observed in protected and unprotected strips. For this reason, we decided to study the relationship between the degree of protection conferred by different concentrations of epinephrine, and the count difference in experimental and control isolated rabbit aortic strips, using ^{14}C -dibenamine hydrochloride as a labeling agent.

Experimental

Rabbit aortic strips were mounted in organ baths as shown in Fig. 4. The response of four aortic strips to epinephrine (1×10^{-8} , 5×10^{-8} , 1×10^{-7} , 1×10^{-6}) were recorded. The strips were then washed with Krebs's bicarbonate solution every 15 min until the tension returned to the baseline. Two of the strips were then treated with epinephrine (1×10^{-6}) for 5 min and ^{14}C -dibenamine hydrochloride (3×10^{-6}) added. After a further 20 min period, the strips were washed three times with epinephrine (1×10^{-6}) and three times with bath fluid. The strips were washed with bath fluid at 15 min

intervals until they relaxed completely (approx 3 hr). Strips treated in this manner were designated experimental strips. As a control, the experiment was repeated with the omission of the protecting dose of epinephrine in the first step. This experiment was repeated four times. The radioactivity associated with the strips was determined as previously described.

These experiments were then repeated using different concentrations of epinephrine as protecting doses viz., 3×10^{-6} , 1×10^{-5} , 3×10^{-5} , and 1×10^{-4} . With each protecting dose, 5 experiments were carried out. Hence, there were 10 experimental strips and 10 control strips for each protecting dose of epinephrine. The results from the above experiments are recorded in Tables III to VII.

Results and Discussion

From the results obtained in Tables III to VII, Fig. 11A-1 was constructed and it is obvious that there is a direct relationship between the protecting dose of epinephrine used and the degree of contractile protection conferred on isolated rabbit aortic strips.

From the results in Tables III to VII, Fig. 11B was constructed showing the relationship between the degree of protection conferred by different concentrations of epinephrine and the count difference in experimental and control strips. It was of considerable interest that as much as 30% contractile protection could be achieved without

TABLE III. THE EFFECT OF EPINEPHRINE (1×10^{-6}) TREATMENT
PRIOR TO ^{14}C -DIBENAMINE HYDROCHLORIDE (3×10^{-6})
INCUBATION OF RABBIT AORTIC STRIPS.

Dry Weight of Aortic Strip (mg)		DPM/mg of Dry Weight of Strip		Remaining Sensitivity to Epinephrine (% of orig- inal)
Control Strip	Experimental Strip	Control Strip	Experimental Strip	
8.0	9.8	320	461	3
7.9	7.3	274	351	1
7.7	8.5	316	305	0
-	9.4	-	303	0
9.3	8.1	341	338	5
10.5	8.3	319	370	8
9.7	9.2	375	291	13
11.7	11.4	335	353	12
10.0	8.7	319	306	0
9.5	8.9	366	248	0
Mean 9.4	9.9	329	333	4.2
S.D. ± 1.1	± 1.3	± 29.9	± 57.6	± 5.1
P Value		> 0.05		

TABLE IV. THE EFFECT OF EPINEPHRINE (3×10^{-6}) TREATMENT
PRIOR TO ^{14}C -DIBENAMINE HYDROCHLORIDE (3×10^{-6})
INCUBATION OF RABBIT AORTIC STRIPS.

Dry Weight of Aortic Strip (mg)		DPM/mg of Dry Weight of Strip		Remaining Sensitivity to Epinephrine (% of orig- inal)
Control Strip	Experimental Strip	Control Strip	Experimental Strip	
10.5	8.9	214	263	55
9.4	8.3	199	234	52
10.0	12.8	261	244	30
8.7	10.6	299	266	50
9.5	10.3	225	228	2
8.9	15.1	223	213	1
8.8	10.5	249	293	8
9.4	10.8	344	254	4
8.2	9.3	283	251	59
10.6	6.8	270	346	40
Mean	9.4	259	260	30
S.D.	± 0.8	± 47	± 25.2	± 23.8
P Value		> 0.05		

TABLE V. THE EFFECT OF EPINEPHRINE (1×10^{-5}) TREATMENT
PRIOR TO ^{14}C -DIBENAMINE HYDROCHLORIDE (3×10^{-6})
INCUBATION OF RABBIT AORTIC STRIPS.

Dry Weight of Aortic Strip (mg)		DPM/mg of Dry Weight of Strip		Remaining Sensitivity to Epinephrine (% of orig- inal)
Control Strip	Experimental Strip	Control Strip	Experimental Strip	
8.2	7.0	290	331	66
7.7	7.7	311	340	75
8.4	9.0	397	237	50
9.8	9.7	319	286	52
9.0	8.1	413	259	59
9.9	9.4	370	319	40
10.7	9.3	282	279	77
7.7	10.4	290	290	73
11.8	9.2	310	308	41
13.7	9.5	297	346	59
Mean	9.7	328	300	59
S.D.	± 1.8	± 47.7	± 35.6	± 13.5
P Value		> 0.05		

TABLE VI. THE EFFECT OF EPINEPHRINE (3×10^{-5}) TREATMENT
PRIOR TO ^{14}C -DIBENAMINE HYDROCHLORIDE (3×10^{-6})
INCUBATION OF RABBIT AORTIC STRIPS.

Dry Weight of Aortic Strip (mg)		DPM/mg of Dry Weight of Strip		Remaining Sensitivity to Epinephrine (% of orig- inal)
Control Strip	Experimental Strip	Control Strip	Experimental Strip	
9.1	10.8	328	306	74
13.0	10.2	329	298	80
9.5	13.9	371	206	78
12.1	12.7	274	286	70
10.2	9.4	411	287	80
10.2	6.3	344	290	78
10.4	12.7	307	277	93
8.1	9.3	292	316	79
8.2	10.3	346	282	82
	9.1		334	70
	9.2		285	70
Mean 10.1	10.3	334	288	77
S.D. ± 1.7	± 1.9	± 41.0	± 32	± 6.3
P Value		<0.05		

Please note: in this group of experiments there are
11 experimental strips and 9 control strips.

TABLE VII. THE EFFECT OF EPINEPHRINE (1×10^{-4}) TREATMENT
PRIOR TO ^{14}C -DIBENAMINE HYDROCHLORIDE (3×10^{-6})
INCUBATION OF RABBIT AORTIC STRIPS.

Dry Weight of Aortic Strip (mg)		DPM/mg of Dry Weight of Strip		Remaining Sensitivity to Epinephrine (% of orig- inal)
Control Strip	Experimental Strip	Control Strip	Experimental Strip	
7.9	11.6	450	305	80
6.1	8.7	422	444	70
7.2	7.4	360	384	67
7.2	9.7	381	371	77
7.5	7.9	267	223	82
9.1	9.3	292	223	90
6.8	6.4	465	368	100
6.0	7.4	489	320	100
8.2	7.8	314	339	91
7.9	8.4	389	260	90
Mean 7.4	8.5	383	324	86
S.D. ± 0.9	± 1.2	± 75	± 72	± 11.6
P Value		> 0.05		

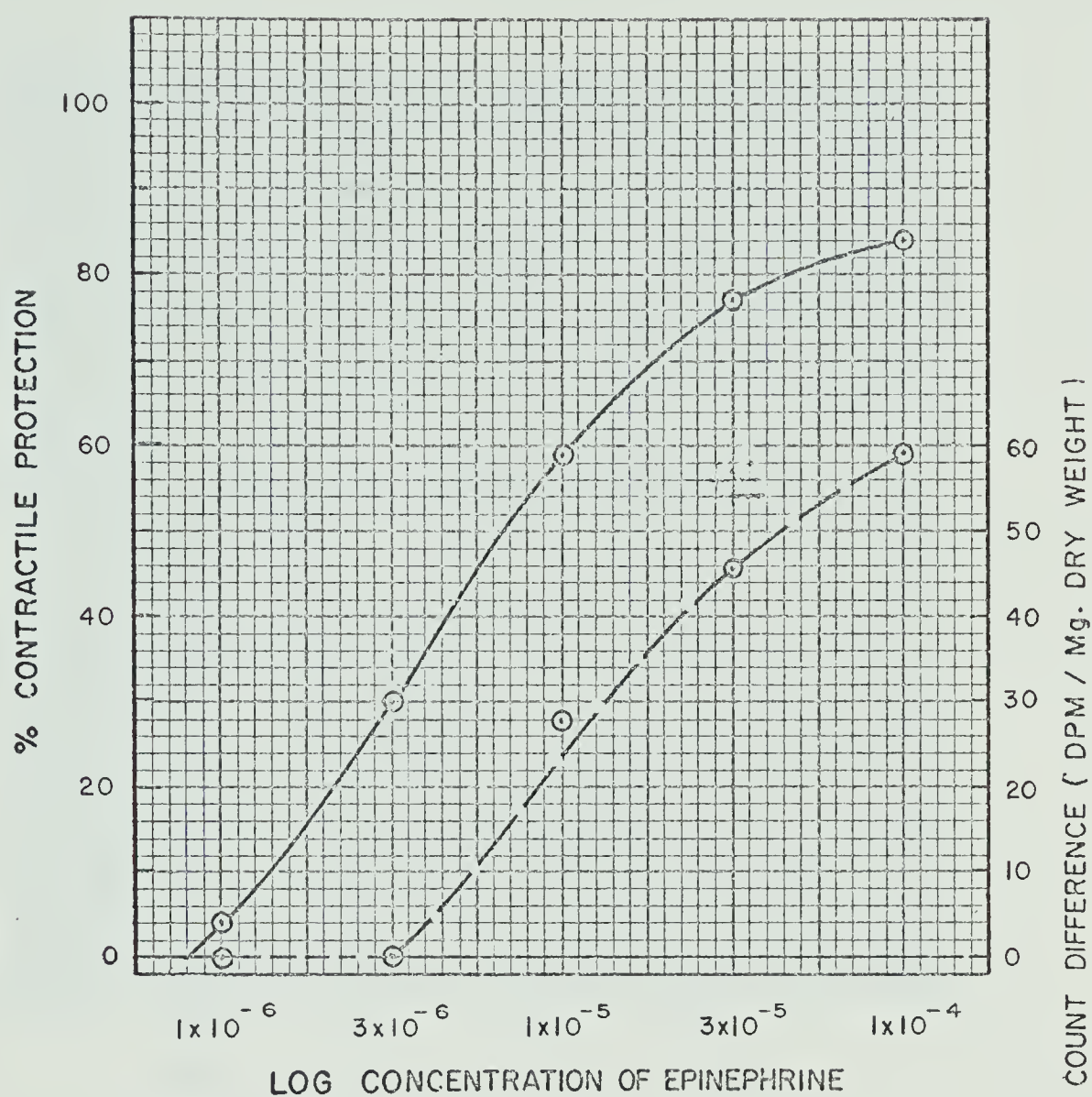


Fig. 11A-1) Relationship between contractile protection observed in rabbit aortic strips and the dose of epinephrine used as a protecting agent.

2) Relationship between count difference (dpm/mg dry weight) in protected and unprotected rabbit aortic strips and the concentration of the protecting dose of epinephrine.

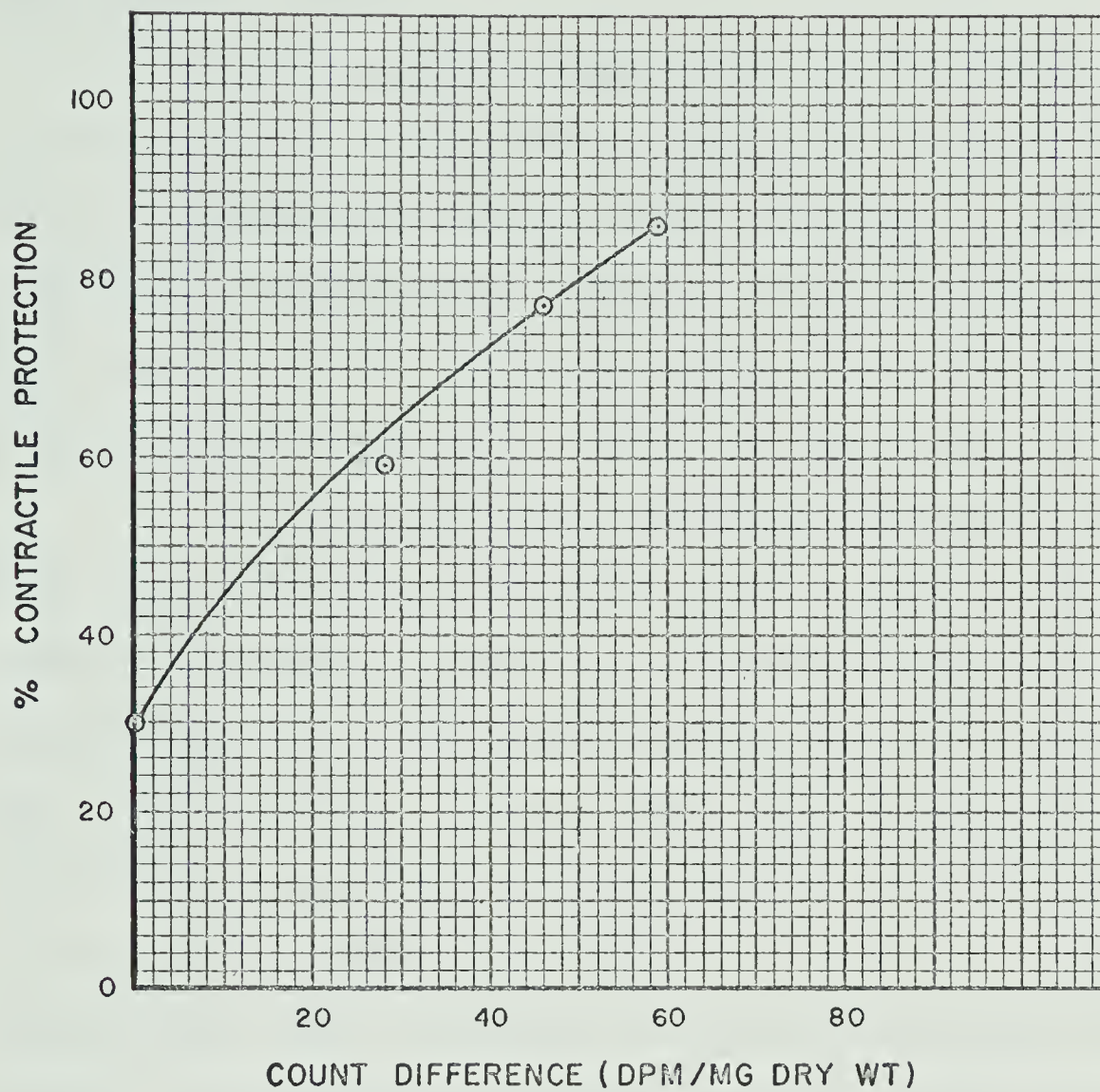


Fig. 11B. Relationship between count difference (dpm/mg weight) between protected and unprotected rabbit aortic strips and contractile protection.

any count difference being observed between experimental and control strips.

From results in Tables III to VII, Fig. 11A-2 was constructed showing the relationship between the protecting dose of epinephrine and the count difference between experimental and control strips. It is apparent that with epinephrine (3×10^{-6}) as a protecting dose, no count difference between experimental and control strips is observed.

The important fact emerging from these studies is that when epinephrine (3×10^{-6}) is used as a protecting agent a 30% contractile protection is conferred on aortic strips without any count difference being observed between experimental and control strips. On the other hand, when epinephrine (3×10^{-5}) was used as a protecting dose, the contractile protection increased moderately to 77% while a highly significant count difference between experimental and control strips of 46 dpm/mg dry weight of aortic strip was observed. Since there are apparently no spare receptors in rabbit aortic strips (Moran et al., 1967; May et al., 1967; Yong, 1968) one would expect a direct correlation between receptor protection and count difference observed between experimental and control strips. In other words, if contractile protection drops from 77% to 30%, one would anticipate that the count difference between experimental and control strips would drop from 46 to approximately 18 dpm/mg dry weight. Since this is not the case it is likely that epinephrine at a concentration of 3×10^{-5} and higher is protecting non-specific

sites as well as α -adrenergic receptor sites from combination with ^{14}C -dibenamine hydrochloride. Thus epinephrine does not appear to combine only with the α -adrenergic receptor.

These studies show that for further advancement in the use of labeling procedures of this type, a receptor protecting agent which is more specific for the receptor than epinephrine is required. Also, these studies show that the concentration of the protecting agent is important. The smallest dose of protecting agent conferring a reasonable degree of protection should always be used to minimize combination with non-specific sites. Yong & Marks (1969) used phentolamine methanesulfonate (3×10^{-6}) as a protecting agent in isolated rabbit aortic strips prior to incubation with ^{14}C -dibenamine hydrochloride (3×10^{-6}). They obtained 100% contractile protection but observed no count difference between experimental and control strips. After using a higher protecting dose of phentolamine methanesulfonate viz., 1×10^{-5} , he observed 100% contractile protection but found a significant count difference between experimental and control tissues. These observations of Yong and Marks (1969), combined with our observations, demonstrate the danger of using a single dose of protecting agent and assuming that the count difference observed between experimental and control strips represents the receptor population. For advances in this field, it is clear that a search for more specific protecting agents and irreversible α -adrenergic blocking agents is desirable. Also, it is necessary to work

with blocking agents of high specific activity since it is possible that significant count differences might be observed where they were not observed in the present study.

Finally, the present study points out the necessity of always studying the relationship between receptor protection and count difference between experimental and control strips irrespective of the nature of the protecting agent and blocking agent employed.

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